

Strategy for leadership in space?

NASA's new strategy for space science offers a splendid promise. But will there be money in the budget to sustain it. And does it matter that it is given in the pursuit of "leadership"?

THE latest forward-planning document from the Office of Space Science and Applications (OSSA) of the US National Aeronautics and Space Administration (NASA) is a cheerful document (see page 767), admirably so in the painful circumstances of the past two years. The most obvious weakness is that many of the plans may not come to pass. Ironically, the projected space station, described in many of NASA's detailed plans as a splendid opportunity, may prove to be a great thief of the funds required for the mostly admirable projects on which the people at OSSA have set their sights. There is also a possibility that a new president next January will rate the danger of a continuing budget deficit more highly than does the present, or that otherwise the overseas lenders who at present keep the US economy afloat will change their minds, cutting NASA's budget and, on past form, OSSA's in particular. And in this hard world, there are few medals for those who make splendid plans which come to nothing.

The essence of what NASA is planning for science and its application is a rounded programme of observation, experiment and development (of instruments and spacecraft). Thus the now long-delayed Hubble telescope is to be complemented by three other "Great Observatories" operating in other regions of the spectrum. Not all of these Earth satellites are new or even recent; the project with West Germany to build the Röntgen X-ray satellite, for example, has been cooking for almost a decade, while the instrument itself will have a useful life of only three years, a fifth the expected lifetime of the Hubble telescope, suggesting that the complementary character of the observations will be fleeting. But NASA should not be crabbed on that account. It is a sign of serious purpose that the agency can think of describing its plans in terms like these. The same is true of what OSSA has to say about its Earth Observing System (not so much a satellite as a sequence of them) dedicated to "understanding the entire Earth system on a global scale ... how its component parts ... have evolved, how they function, and how they may be expected to continue to evolve on all timescales". This is heady stuff, but none the worse for that. There are many who will be hoping that the plans come true.

False notes

The false notes in OSSA's planning document are in a different key. In one of several independent and even orthogonal statements of objectives for the future, OSSA lists alongside "complete ... the detailed scientific characterization for virtually all of the Solar System" this joker — "determine, develop and exploit the unique capabilities offered by the space station ... to study the nature of physical, chemical and biological processes in a microgravity environment". The most obvious discord is that the two objectives differ so much in their content that they do not belong in the same list.

The first, ambitious though it may seem, is a grand concept. "So now", people will be saying, "we really know the Solar System." Meanwhile, there will be another crew of investigators bent on standing the process of investigation on its head, looking for a purpose for the tool that US policy will have thrust into its hands in the first half of the next decade — a space station whose

justification, in the US space policy promulgated in January, is simply to "establish a permanently manned presence in space". It is as if Columbus, embarking on his great journey to the new world 500 years ago, were to have offered his sponsors on the side to exploit the potential of seashells for making ornamental necklaces. (That he may have done just that is beside the point.) One purpose, attained or not, of the kind that dignifies what people do, the other is part of a conspiracy to show that the space station is not just show.

Space leadership

There is worse to come. NASA's strategic plan is subtitled "A Strategy for Leadership in Space through Excellence in Space Science and Applications". That jives well with President Reagan's statement on 5 January that "a fundamental objective guiding US space activities has been, and continues to be, space leadership". OSSA's document goes on to explain that this entails both "a vital scientific and applications program" and its "visible and significant achievement". This does not quite mean that space science is regarded as the unique means, or even as exclusively one means, of achieving leadership, although the space research community would be well advised to note the language. But it does imply that the delectable menu of launchings laid out for the rest of this century has something of the quality of an accident.

Space science is a vehicle suited to the pursuit of leadership because, with nearly three centuries of maturation since Galileo's telescope, and otherwise being harmless, it happens also to be in good shape. It is not unfair to say that space science is the adventitious beneficiary of the pursuit of leadership, and it would not be the first time that useful progress has been sustained by indulgent patrons. Helping to define a role for the space station will not be all that demeaning. But the environment of the new promise should be clearly understood.

Whenever will it be feasible to turn this argument around, putting space science first and accepting that leadership is not a commodity of a kind that can be manufactured, or even a commodity worth having if that is its appearance? The truth is that the United States has won much honest admiration around the world for what NASA and its associates have done (Pioneer spacecraft, countless Voyagers, weather satellites and inestimable benefits such as telecommunications satellites). Would not more of the same have done as well as the explicit pursuit of leadership? Those who exercise leadership in the performing arts, or in the education of the young, or even at playing tennis, do so not because they have sought leadership, but because they do these things well. To be fair, OSSA's strategy document does artfully attempt to put the case, by reflecting (as far as the staccato note-form style of its document will allow) on the benefits of knowing whether the Universe will continue to expand or not, or by raising the issue of living things elsewhere within it. But it is a half-hearted attempt at civilizing a program so vast, and so dependent on financial hazards not yet apparent, that the pursuit of leadership by sticking with OSSA's own declared objectives may have seemed dangerous.

One of these days, with another president (not necessarily the



next), and in a different climate, it may be easier to tell what space science is for: like the rest of science, it is for understanding part of what the natural world is all about. To say this is not to say that science has no practical function (because the opposite is true), but merely that the more usual practical benefits are often accidental. Leadership, whatever it may mean, may be among them, but is usually a second-order accident. □

Is 1992 already here?

The European Commission is behaving as if Europe were already a single market.

THE European Communities, often mis-named "the Common Market", seems to have had a rush of rich red blood to the head in the past few weeks. First, having last year warned European airlines that they would not be able indefinitely to continue their cosy cartel arrangements with each other, the European Commission has now written to them individually saying in clear terms what steps they will have to take to make legal their arrangements for restricting seat capacity on certain routes (and for sharing the revenues that result). Although the commission has not yet chosen to take the fateful step of saying that, at some predetermined stage, it will be illegal for a government to subsidize an airline, whereupon the true benefits of civil aviation technology will accrue in Europe as they already have elsewhere.

Meanwhile, the European Commission appears to be going after the telecommunications and broadcasting industries, even bigger and more promising fish for reform. The immediate issue concerns the standardization of the whole of the European industry: should there be a cellular radiotelephone network for the whole of Europe and, if so, by what standards should it be built and to whom should the work be contracted? The commission has a special brief under the terms of the Treaty of Rome to promote cross-cultural or transnational activities, and may fairly consider mobile cellular telephones to be one of them. The result is that the commission is busily encouraging the standards by which the same mobile telephones might be made to function in places as different as France, Greece, Holland and Ireland. More daring still, it is also talking to manufacturers in almost-open committee meetings about their capacity to supply all that equipment, or to operate pan-European networks.

The good news is that this attitude appears to be infectious, for technical as well as psychological reasons. There is no point in owning a mobile Eurotelephone, incurring in the process the obligation to pay an annual membership fee, without being able to call up people not already as privileged. So the commission, no doubt encouraged by the support of the people who reckon they would make fortunes from building even a fraction of the new equipment, has taken to saying that its scheme will not succeed unless cellular Eurotelephones have access to the telephone lines at present owned and operated by the great public monopolies, of which the Deutsches Bundespost is the largest, the most conservative and the most expensive. But everybody knows that right of access to a monopoly's assets is the death of that monopoly. (It might well be cheaper for West Germans to carry mobile telephones in their pockets than to use their monopoly's connections between their fixed instruments.) Those who relish the maxim that the grandest are those who have the furthest to fall may confidently await the ending of that anachronism.

Protectionism

The snag is that a long time may have to pass. The European Commission is just now buoyed up by its belief that "everything" will be different after 1992; much will be, but not everything. Moreover, the commission's own case is weakened by its Eurochauvinism. While the commission is talking to European manufacturers about the supply of new telecommunications

services, it cold-shoulders manufacturers from elsewhere (not unconnected with the fact that the US House of Representatives passed another restrictive trade bill last week). Japanese companies are among other potential suppliers. Are they being given a chance to make their pitch and, if not, why not? Even from a chauvinist point of view, the commission should pay some attention to the question of whether it seeks efficient services so that Europeans can do other things, or whether it needs the business (in which case, agriculture might have to go to the wall).

Broadcasting is more difficult and contentious. The commission's theoretical position is that it is required to promote pan-European standards (relative decibels between transmitters and receivers, for example, or how many minutes of advertisements there should be in any hour of television broadcasts), while nursing its predilection for competition. The commission would be well advised to acknowledge that its gargantuan two-volume consultation document on the issues, published two years ago, was read by very few and understood only by a minority among them. The trouble with television (as distinct from telephones) is that different kinds of people take it to heart. If the European Commission is determined to behave as if 1992 were here already, it had better be discriminating about the causes for which it would go to the stake. □

Money problems multiply

A popular view of the world's money problem is that nothing much has changed. Is that correct?

"STEADY as she goes" is an old seafarer's saying most often intoned with particular urgency when ships are about to hit the rocks. The irony is nicely embodied in the communique put out by the Group of Seven (familiarily known as G-7) at the end of its meeting in Washington some three weeks ago; it can have been only cruel bad luck that it should have announced that all is well with the world's finances on the eve of the announcement that the US trade balance had unexpectedly worsened. Things have been in turmoil ever since — but they had been in turmoil for some time before that, so that the most recent events make very little difference.

Enough has been said about the US federal government's domestic deficit, which cannot in any case be reduced before the US elections early in November. Events could go awry between now and then, but only in such a way as to shuffle people's electoral chances. The most effective cause would be a second poor month, but that is against the odds; it is unthinkable that such a cheap dollar would not help to sell dollar-denominated goods.

The substantial worries for the next half year lie elsewhere, and are most prudently put as questions. Japan, suddenly discovered to be the most prosperous nation on the surface of the Earth, remains a curious mixture of conservative agrarian and devil-may-care urban sustained in its expectations of the future only by its cheerful experience of the past quarter of a century; can that last? West Germany, once the West's miracle of enterprise, seems to have lapsed into book-keeping; is that intentional and, if so, who intends it? Britain, quite recently the sick man of Europe, chose to beat double-digit inflation by being tough and is now assiduously running a supply-side economy by fiscal devices for allowing house prices to leap ahead against the wishes of the Chancellor of the Exchequer; just when will those chickens come home to roost? The fact that the poor countries are even more short of funds than before the West's banks became prudent, declining to add to their loan books, was easily predicted; but does this mean that there is now no hope for the people now thoroughly ground down? That there is continuing trouble in the Middle East is almost neither here nor there. What the world needs is a way of running its finances with ingredients other than illusions. □

NASA's space science ambitions looking costly

- Space station strains the budget
- Astrophysics seen as the key

Washington

By its own admission, the Office of Space Science and Applications (OSSA) at the National Aeronautics and Space Administration (NASA) is in a period of transition. The exhilarating rapid-fire pace of the 1960s has given way to a period when missions are more complex, require more planning and place greater demands on resources.

To direct NASA's future course, Lennard Fisk, associate administrator for space science, has come up with a strategic plan that will "serve as the basis for OSSA program planning in the future". The plan, providing clear goals and means for achieving them, is still predicated on a difficult premise: that an increasingly chary Congress will continue to provide enough money to pursue continuing projects let alone start new ones.

The immediate future for space science at NASA is rosy. With a modicum of good luck, next year will see the launch of the Cosmic Background Explorer, the Magellan Venus radar mapper, the Hubble Space Telescope, the Astro Spacelab mission, the Galileo mission to Jupiter and the Voyager 2 encounter with Neptune. But after that, both new money and new directions will be needed.

Fisk's plan proposes the pursuit of world leadership in space science through an initiative in astrophysics. Fisk is proposing four 'Great Observatories' to give the United States observing capability in all major wavelengths. With the Hubble Space Telescope as the centrepiece, the spacecraft will include the already started Gamma-Ray Observatory, the Advanced X-Ray Astrophysics Facility, a new start in President Reagan's 1989 budget request to Congress and a Space Infrared Telescope Facility still on the drawing boards.

The observatories fall into the category of major missions that form part of the strategic plan. Other major missions would include the Comet Rendezvous Asteroid Flyby mission, the Cassini mission to Saturn, an Earth Observing System to

fly aboard the space station's polar platform, and a solar probe.

Fisk's hope is to start one of these major missions each year. But the plan also calls for starting at least one small mission each year. Such missions might include Earth probes such as the Magnetic Field Explorer and the Tropical Rainfall Measurement Mission or Lifesat, a reusable spacecraft for life sciences experiments.

These small missions "are particularly important for the training of the next generation of scientists and engineers", because they can be performed on a small enough scale to be managed by a single university and should take about the time needed to earn a graduate degree.

The plan also declares that "it is time to move aggressively, but sensibly" to develop projects to take advantage of the space station. Six pieces of equipment are specifically proposed: a furnace facility, a modular combustion facility, a fluid physics/dynamics facility, a modular containerless processing facility, an advanced crystal growth facility and a biotechnology facility. The plan also proposes strengthening NASA's research base over the next five years.

The Fisk plan attempts to provide rational priorities to a scientific programme, and many agree that it succeeds in that attempt. But reality may still force decisions to be made on a budgetary rather than a strategic basis. All the proposals are based on a planned growth in the NASA budget. But the costs of the space station are such that Congress will have either to provide NASA with substantially more than the approximately \$11,500 million it is requesting for this fiscal year or see other programmes, including space science, dry up.

Fisk's plan provides a statement of where NASA would like to find itself by the start of the next century. It is probably a necessary step to achieving the leadership role the plan seeks, but without money it will not be a sufficient one.

Joseph Palca

Leaked report on Star Wars

Washington

THE Congressional Office of Technology Assessment (OTA), after two years of study, has concluded that any system designed to fulfill President Reagan's vision of "Star Wars" defence against Soviet missiles would probably fail "catastrophically" should it ever be used. The two principal failings of the Strategic Defense Initiative (SDI), says the OTA, are that countermeasures can be easily devised which are simpler and cheaper than the defensive system, and that battle coordination by computer would require software of such complexity that it could never be tested to assure the needed reliability.

The report was finished seven months ago, but since then has been mired in the declassification process at the Department of Defense (DoD). OTA staff members have not hidden their dissatisfaction with the delay. Last Friday, a copy of the summary chapter of the report's findings was circulated to OTA's Congressional board, and an unauthorized copy was obtained by the *Washington Post* in time for its Sunday edition.

Three chapters of the OTA report are still classified, although the remainder has been cleared by the DoD for public release. OTA still hopes to make the whole report public next month, but may decide to release only the nine chapters which have been cleared rather than put up with further delay. Last year's report by the American Physical Society on the feasibility of SDI beam weapons languished similarly (*Nature* 326, 815; 1987), and was then criticized by SDI supporters for omitting recent developments.

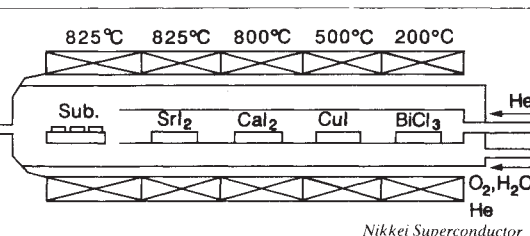
Neither the DoD nor the SDI Organization has made any specific response to the criticisms, saying they will have a full reply ready when the whole report is made public. But Secretary of Defense Frank Carlucci has said that the OTA report is tantamount to asserting, ten years before helicopters were invented, that helicopters were "doomed to failure".

Many critics have pointed to survivability and battle control as the weakest parts of SDI, but John Pike, of the Federation of American Scientists, believes the OTA report will carry special weight because it is the first comprehensive study of SDI as a complex system rather than a collection of gadgets. Little has been achieved in these crucial areas, and SDI research contracts have been numerous and small, an indication, says Pike, that many possibilities are being studied but few are showing promise. Coming at the end of President Reagan's term, the OTA report may be enough to shatter the Star Wars dream.

David Lindley

Superconductor methodology

A method for cheap mass production of superconducting wafers may result from Fujitsu's new vapour deposition technique involving a temperature gradient. Substrate is MgO. See page 770 for details.



OECD wants more science cooperation

Paris

INTERNATIONAL sharing of scientific and technological advances is good in principle but difficult to achieve in practice. This was the conclusion of science and technology ministers when they met under the aegis of the Organisation for Economic Co-operation and Development (OECD) last October. Now, the OECD Council has drawn up a list of recommendations aimed at overcoming obstacles to greater international cooperation in research.

According to a recent OECD communiqué, the increasing contribution of private enterprise to basic science and technology research in developed countries, although advantageous on a national level, brings with it an unwillingness to share findings unless copyright and patent rights are respected. Unfortunately, not all OECD member states subscribe to international codes of conduct designed to protect these rights.

The OECD recommends three major ways to improve the flow of information across national boundaries: removal of trade barriers; promotion of basic research and dissemination of findings; and increased international cooperation through joint research programmes.

Specifically, the council recommends that member countries promote advances in scientific and technological knowledge by supporting basic research and maintaining up-to-date research facilities; promoting the education and advanced training of future generations of scientists, engineers and technical personnel and facilitating the exchange of students and scientists between member countries; facilitating the international mobility of scientists and engineers, and in particular their access to major basic research facilities; promoting the dissemination of the results of basic research, particularly through publication in internationally available scientific literature, access to databanks and networks, and open participation in scientific meetings.

So-called 'developing' countries are often at a disadvantage, not only because of limited facilities for advanced research, but because access to computer networks may be non-existent or restricted.

To tackle the problem of technology transfer, the council not only recommends the "circulation, exchange and trade of technologies", but also the promotion of "improved universal protection of intellectual and industrial property rights". The difficulty here, as experience has shown, is that it is often impossible to sanction organizations that flout these rights in countries which do not recognize international copyright and patent laws.

Peter Coles

Science research a key issue in French electoral campaigns

Paris

"If France wants to succeed, research will have to become the Republic's favourite child", says President François Mitterrand, in the run-up to the May presidential elections. Mitterrand's adversary, neo-gaullist Prime Minister Jacques Chirac, agrees, but the policies (and track records) of the two could not be more different.

By bringing the issue of public-sector spending on research to the forefront of his electoral campaign, Mitterrand has embarrassed Chirac. When the socialists lost power in 1986, Chirac's government set about dismantling the country's public-sector research infrastructure. Over half of the 'savings' in public spending were found within the research budget, annual recruitment, and promotions within the Centre National de la Recherche Scientifique (CNRS) were suspended, and ANVAR, the government agency promoting technology transfer from universities to industry, was threatened with closure.

Chirac would have preferred not to be reminded of this 'Dark Age' period. Instead, he claims increases in state spending on research of 8 per cent in 1987 and of 10 per cent in 1988. But the increases are only apparent when defence and civil research spending are lumped together. If the extra FF33,000 million (£3,200 million) allocated for defence research is ignored, spending on civil research has increased by only 2.3 per cent since 1986 which, with inflation, amounts to a slight net decrease.

Similarly, Chirac can point to the creation of 150 new research posts in the public sector this year. But 288 posts have been suppressed, while the temporary suspension of CNRS recruitments in 1986 and 1987 makes it difficult to see what the real annual trend is.

If Chirac's initially draconian attack on public-sector research was halted when, in late 1986, his research minister, Alain Devaquet, was forced to resign in the face of mounting unrest in universities and research centres, the renaissance of research as a government priority has been founded on a demand for more support from industry. According to Chirac, the private sector will need to contribute an extra FF25,000 million towards research by 1995 if France is to keep up with foreign competition in technology.

To stimulate industry's share of the research bill, the present research minister, Jacques Valade, has voted FF500 million of annual tax incentives, industry-based research scholarships and new mechanisms for collaboration between private and public-sector scientists.

Mitterrand also wants to see France

increase its research spending, but his electoral campaign, published in full in the French daily newspaper, *Le Monde*, last week, is vague when it comes to concrete measures to achieve this. Between 1979 and 1985, public sector spending on research increased from 1.81 per cent to 2.25 per cent of gross domestic product (GDP), the socialists having continued a trend started under Raymond Barre's conservative government. Mitterrand feels that a figure of 3 per cent of GDP should be aimed for by 1992. Although it was the socialists who introduced a system of tax incentives for industrial research, it is within state-supported research that he



places his faith, precisely because industry "is not pulling its weight".

Under the present government, the emphasis is firmly on research with clear short-term commercial applications. Mitterrand, however, sees the role of basic research as fundamental. "Let us neither be afraid of nor criticize research that finds nothing. Out of the mass of investigations springs discovery, the research that finds." Mitterrand cites research to sequence the human genome, which will "cost less than a trip to the Moon". Yet, he adds, "no country in Europe devotes a serious part of its budget" to this quest.

Mitterrand has not been slow to remind voters that it was he who, launched the European Eureka technology research initiative, as an alternative to joining the US 'star wars' research programme.

Chirac has called Mitterrand's rhetoric "woolly" and "misinformed", but it has been effective — the major daily newspapers have given considerable space to the *volte face* in Chirac's research policy. If Mitterrand is re-elected on 8 May, as public opinion polls and his success in the primaries suggest will happen, he will still have to contend with a right-wing majority in government. He would undoubtedly want to name a new prime minister but he could find it difficult to reintroduce the public spending increases so unpopular with the conservative majority. **Peter Coles**

Another report smiles on human genome sequencing project

Washington

CONGRESS'S own research agent, the Office of Technology Assessment (OTA), last week produced at a congressional hearing a reflective report* on the project to map the human genome. While applauding the project, the report differs from many other on the topic in exploring hitherto neglected ramifications of the project.

Although the OTA effort began life as the 'human genome project', the report makes it quite clear that other than human species will ultimately play a role in understanding the genetic information gleaned from mapping projects.

OTA also seeks to defuse the contentious notion that a genome project would be solely concerned with constructing a complete sequence of the 3,000 million nucleotide base pairs that make up the human genome. Although this may be an ultimate goal, the OTA report points out that physical maps, genetic linkage maps, clone repositories and genetic databases will all become available as a result of the project, and will have immediate utility for the biological sciences.

The report also says that genome projects are not in the same category as 'big science' projects such as the Manhattan Project or the Apollo programme to land a man on the Moon. The component parts of genome projects do not require the same scale of spending, and the technical goals are not nearly so sharply focused.

Congress will probably find the section in the report on organization the most useful. In unusually forthright terms, the report says that the appointment of a single lead agency to run the project would be fraught with problems. OTA warns that the process of selecting a lead agency "would delay progress and diminish overall funding".

Instead, OTA seems to lean towards an interagency task force to coordinate the various efforts of the National Institutes of Health, the Department of Energy and the National Science Foundation, each of which would support some component of the project. In this respect, OTA differs from the National Research Council's report on the human genome project, which advocated a single lead agency (see *Nature* 331,467;1988).

The report also raises some difficult issues relating to patents, copyrights and technology transfer that will arise as private companies and foreign governments join federally supported research laboratories in working on the project.

The report also says that there will be

difficult ethical and social problems. For example, genetic mapping will undoubtedly provide more effective diagnostic tools for the detection of disease, but diagnostics seems inevitably to precede the development of effective therapies. Eugenic issues will also need attention as a better picture of genomic organization emerges.

The lack of progress in resolving these issues has prompted biotechnology critic Jeremy Rifkin to form a coalition pushing for more congressional attention to the social ramifications of genome projects. Rifkin's coalition, drawn from a wide spectrum including ethics specialists, labour leaders, members of women's health organizations, insurance watchdog groups and disabled rights groups, intends to work for a human genome policy board to advise Congress on the implications of genome research.

Congress has already taken up this issue (see below), but so far not especially effectively. Rifkin says he has no objection to spending money on mapping and sequencing efforts, so long as public concerns about the project are heard.

LeRoy Walters, director of the centre for bioethics at the Kennedy Institute of Ethics and chairman of the OTA advisory panel on mapping the human genome, agrees that scholarly ethical research must proceed in tandem with biological work. Otherwise, Walters says, ethical problems will be upon us before we have decided how we want to cope with them.

Joseph Palca

■ In typical bureaucratic style, Congressional interest in the mapping and sequencing of the human genome has spawned proposals to create panels and review boards to evaluate and manage the project. Legislation to focus the organization of the project and direct its role in enhancing national "competitiveness" has been entered in both houses of Congress. But what of ethical issues?

To fill the gap, plans have been laid in Congress to resuscitate a Biomedical Ethics Board originally set up in 1985 to wrangle with issues such as fetal research and genetic screening. The board of six Senators and six Congressmen has been languishing for the past two and a half years, divided over the composition of its advisory committee and its stance on the issue of abortion. The selection of committee members was completed three weeks ago.

Whether the board finally will get off to a start by grappling with the ethical questions posed by the human genome project remains to be seen.

Carol Ezzell

SE Asia drug centre

THE World Health Organization (WHO) has established a centre on drug information at the Central Drug Research Institute at Lucknow in India. The centre, the first of its kind established by WHO, will in the first instance run for four years. It will act as a focal point for information on drugs for the 11 states that comprise the South East Asia region. The centre will develop and maintain an information base on sources of drug manufacture, prevailing prices and registration, and patent status. In addition, it will train WHO fellows in aspects of drug information services.

Of particular importance will be the centre's role in helping member states to decide whether to register drugs — several developing countries have not yet established effective mechanisms for drug registration, which has led to allegations of unscrupulous marketing by pharmaceutical manufacturers. S.H.

Universities win battle

THE Cape Supreme Court has dismissed with costs an application for leave to appeal against the setting aside of subsidy conditions imposed on the University of Cape Town (UCT) by the South African government last year. On the same day, the state withdrew an application for leave to appeal against the setting aside of identical conditions imposed on the University of the Western Cape (UWC).

Delivering judgement on 31 March, Mr Justice Howie said that there was no reasonable chance that such an appeal would succeed. In February, the court ruled that the conditions imposed on both UCT and UWC were invalid, and last month the Natal Supreme Court made a similar ruling for the University of Natal.

This means that the universities have won the legal battle to have the regulations declared invalid, as the case cannot be referred to the Appeal Court in Bloemfontein. The prospect remains that Minister of National Education F.W. de Klerk may decide to put legislation before parliament to effect the conditions he wants. M.C.

ACOST speaks out

ACOST, the British government's Advisory Council on Science and Technology, has made a rare public announcement. Following a meeting earlier this month chaired by the Prime Minister, Mrs Margaret Thatcher, the council last week issued a statement that it has commissioned the Natural Environment Research Council (NERC) to prepare a report on the "extent and effectiveness of involvement by the UK in research on the global environment" and "the importance of global environmental research to the UK for government, economy and society". The council also wants to examine the potential for industry to exploit environmental research. S.H.

*Mapping our Genes: The Genome Projects: How Big, How Fast? Office of Technology Assessment, Washington DC, 1988.

Human trials go-ahead for the AIDS vaccine with a difference

London

A PROTOTYPE AIDS vaccine that has been awaiting approval in the United States for a year has received the go-ahead in Great Britain. A small-scale trial of the toxicity and immunogenicity of the vaccine, a synthetic peptide, will begin this summer at St Stephen's Hospital in Fulham.

The 30-amino-acid peptide, known as HGP-30, represents a small part of the gp17 protein of human immunodeficiency virus 1 (HIV-1), the cause of AIDS. Since peptides are not on their own very immunogenic, the HGP-30 has been linked to a carrier, keyhole limpet haemocyanin, and will be given together with alum as an adjuvant.

Initial tests, which are expected to involve 24 volunteers, will examine potential side effects and the extent to which the preparation induces antibodies that will neutralize HIV-1. Tests on animals, including rodents, rhesus monkeys and two chimpanzees, have already demonstrated the safety and immunogenicity of the preparation.

The tests will be carried out by Dr Brian Gazzard, with the approval of the Riverside Health Authority and provisional approval of the Clinical Trial Section of the Department of Health and Social Security. Viral Technologies Inc., a Californian joint venture of the CEL-SCI Corporation and Alpha 1 Biomedicals, will supply the HGP-30 preparation.

HGP-30 represents amino acids 86-115

of the p17 protein, with one amino acid difference. While, unlike the envelope protein on which most candidate vaccines are based, p17 is not generally considered to be on the surface of HIV-1, infected patients nonetheless produce antibodies to it. Recent evidence suggests that in many patients the disappearance of antibodies to p17 is a prelude to the development of AIDS. So a vaccine that induces such antibodies may help prevent progression. Therefore it is likely to be tested first in those who are already infected with HIV-1.

Much of the work that led to the trial has been carried out by a team led by Professor Allan Goldstein of The George Washington School of Medicine and Health Sciences in Washington D.C. together with Dr Prem Sarin and colleagues at the National Cancer Institute in Bethesda.

Earlier this week, Goldstein expressed some frustration at the lack of decision by the Food and Drug Administration, which has been considering an application for a trial of HGP-30 at the George Washington University for a year. He was delighted, however, that the UK trial is to begin and hopeful that applications in other countries will also receive approval.

Other trials under way include that of a killed HIV (see *Nature* **332**, 668; 1988), a recombinant vaccinia virus that produces the gp160 envelope protein of HIV (see *Nature* **332**, 728; 1988), and purified gp160 itself.

Peter Newmark

No rare earths in new film

Tokyo

THE speed of research on the new rare-earth-free superconductors is breathtaking. It is barely a month since Tohoku University researchers announced a chemical vapour deposition technique for making thin films of yttrium-barium-copper oxide (*Nature* **332**, 295; 1988), but Fujitsu Ltd already claims to have used a similar technique to deposit an epitaxial monocrystalline thin film of bismuth-strontium-calcium-copper oxide on a large-area substrate. The technique has potential in the mass production of high-critical-temperature superconducting epitaxial wafers and electronic devices.

Chemical vapour deposition is a cheap and versatile technique widely used in the semiconductor industry for production of thin films, but it is only recently that Japanese researchers have adapted the technique to high-temperature superconductors.

In Fujitsu's method, halides of bismuth, copper, calcium and strontium are heated in a quartz vessel under a gradient of temperatures from 200°C for BiCl₃ at the downstream end of the vessel to 825°C for SrI₂ at the upstream end (see diagram on page 767). Inert helium carries the vaporized halides towards the substrate (MgO), where chemical reactions occur in an atmosphere of oxygen, helium and water vapour, and a superconducting thin film is deposited on the substrate. No annealing at high temperature is required.

Using the technique, Fujitsu claims to have coated a 30 × 30 mm basal crystal plane (001) of MgO with an epitaxial monocrystalline film of bismuth-strontium-calcium-copper oxide. The film is 0.3 micrometres thick, was deposited in about half an hour, and has a critical temperature, at which electrical resistance drops to zero, of 78 K.

This is the first convincing report of a monocrystalline thin film of the new rare-earth-free superconductors. Initially Fujitsu plans to develop the technique to deposit films on six-inch silicon wafers. And in the short term, the company foresees application of the technique to the production of a superconducting quantum interference device (SQUID) for measuring minute magnetic fields.

In the long term, development of multi-layer deposition may allow manufacture of tunnelling-type Josephson devices, superconducting transistors and large-scale integrated circuits with superconducting wiring. But Fujitsu still has a long way to go — the critical current density of the thin film has not yet been determined, according to a company spokesman.

David Swinbanks

Lenin prizes reflect centralized Soviet science

London

THE Lenin prizes for Science were announced on 22 April, the anniversary of Lenin's birth. This year, nine prizes were awarded, in line with the revised procedures introduced under the *perestroika* reforms, which reduce the number of prizes and makes the selection procedure more rigorous. Of these, seven were given for fundamental research (three for physics, one for geophysics, one for neuroscience and one for linguistics) and only three for work with obvious practical applications — the synthesis of carbohydrates, automatic control and a pipe contact-welding process.

Only one prize (for the welding process) is shared between academics and industry, and seven out of the nine prizes go to scientists from the European part of the Soviet Union. Only one prize, for tectonics, went to the far-eastern branch of the Academy of Sciences. And seven out of the nine prizes went, wholly or partly, to academicians or staff of the Academy of Sciences of the USSR, while the remaining two went to the

Georgian and (in part) the Ukrainian Academies. Soviet science, the prizes suggest, is still heavily dependent on the old, traditional centres and structures.

A very different impression, however, emerges from the celebratory articles for "Scientists' Day", the third Sunday in April. These commend, not the long "cycles" of work which gain the Lenin prizes, but the recent advances in superconductivity and the new cryogenic aviation fuel. They stress the outlying science centres, the far eastern and Ural branches of the Academy, where science in the words of *Pravda* "is coming of age". The contributions of researchers in higher-education colleges and industrial institutes stress the need for closer links between science and production, and the need for science to conform to the new principles of cost-efficiency. In short, a summary of Mr Gorbachev's vision of Soviet science. But, the Lenin prizes suggest, this is not yet where the honours are to be won.

Vera Rich

UK research centre programme gathers speed despite doubts

London

PROPOSALS to set up a series of interdisciplinary research centres (IRCs) within Britain's higher education system continue apace despite a deep undercurrent of scepticism from many rank-and-file researchers. The government has given its seal of approval to the concept, which is being vigorously pursued by the four principal scientific research councils. The Advisory Board for the Research Councils (ABRC), which gives the government guidance on how the science budget should be divided, was due to meet earlier this week to consider draft proposals from each of the research councils for the next batch of centres. So far, four centres have been given the go-ahead, all under the auspices of the Science and Engineering Research Council (SERC). In its forthcoming public expenditure bid, the ABRC is expected to ask for sufficient extra cash to start about a dozen further centres.

The Agricultural and Food Research Council (AFRC) earlier this year invited institutions to bid to host centres from a list of eight possible areas of research. A shortlist of three priority areas has now been arrived at: animal transgenic biology, plant molecular biology and, with the Natural Environment Research Council (NERC) and the Economic and Social Research Council, agricultural environment and land use (the subject of a failed £5.7 million programme bid last autumn).

NERC will be seeking some £20 million to relocate the Institute of Oceanographic Sciences Deacon Laboratory in Surrey to the University of Southampton to create an IRC in deep-sea oceanography. Six more IRCs have been proposed, including, in common with SERC, one for atmospheric sciences (the bids are separate).

The Medical Research Council has been given approval to relocate its toxicology unit from Carshalton in Surrey to form an IRC at an as yet unspecified university site. The MRC has put in three further bids, for cell biology, in London; macromolecular engineering, probably at Cambridge; and therapeutic immunology, for which a location has yet to be agreed.

Since announcing its first four IRCs, SERC has received some 300 suggestions for further centres. Plans for a centre in novel semiconductor materials at Imperial College, London, are likely to get final approval in June. SERC's first centre, in superconductivity, based at Cambridge and announced in December, has yet to open. Discussions are continuing between the university, the University Grants Committee and SERC on financial and administrative details. The centre will be officially inaugurated on 21

June. Until the centre receives its money, a director cannot be appointed.

Despite the enthusiasm of the research councils, doubts about the wisdom of the IRC concept remain. The Association of University Teachers has expressed "grave concern" that the centres could "move all too readily towards establishment as private autonomous institutions", undermining the quality of teaching at the host institutions. The other chief concern is that the centres represent an unhealthy move towards too much directed research with little room for creativity and innovation.

Worries also persist about the speed with which the programme is moving when fine details on such aspects as the centres' management apparently remain to be resolved. It was only last July that the idea for IRCs received a full airing, when the ABRC published its now

notorious discussion document, *A Strategy for the Science Base* (see *Nature* **328**, 280; 1987). One factor in the equation that ABRC had envisaged, but which has been overtaken by the speed of events, is the role of the new Centre for Exploitation of Science and Technology (CEST). Launched last November and financed mainly by private industry (see *Nature* **330**, 196; 1987), CEST has yet to move into permanent premises and recruit its full complement of ten staff. It has not been involved in selecting the present list of IRCs being considered by the ABRC. Chief executive Bob Whelan is nevertheless confident the CEST will have a central role to play in the selection of topics of importance for future IRCs. Whelan said this week that CEST would aim to develop a close relationship with the individual research councils. He also injected a note of caution into the process of deciding which topics to pursue. "I want to avoid snap judgements — selection of areas has to be based on robust data, well-argued and logical."

Simon Hadlington

Minister champions European cause for West German research

Bonn

ANNUAL budget squabbles notwithstanding, West German Research Minister Heinz Riesenhuber takes much of the credit that support for basic research in West Germany has increased steadily during his six-year tenure of office. The percentage of the Ministry's budget devoted to basic research has increased from 26 to 39 per cent during that stretch.

Riesenhuber now says he is trying this year to give basic research a European dimension. As president for six months of the European Community (EC) Council of Science Ministers, he is concentrating on defining European participation in the Japanese Human Frontiers Science Program and on uniformity bioethical standards in the European Communities.

Just days before the next international conference on Human Frontiers, Riesenhuber reduced the possible goals of the still-nebulous programme by one. Sequencing the human genome would be "too technocratic" for Human Frontiers, he says.

Within Europe, concern is growing about the implications of the big step towards integration planned for 1992, when the twelve member countries have to agree on norms in education and production. Riesenhuber had comforting words for West German researchers, saying there would be "no caesura" for them in 1992. Scientific exchange is already quite successful between West Germany and smaller European states, although

Riesenhuber believes that exchange with France and Great Britain could be improved.

Riesenhuber's remedy is not only to concentrate university studies on the essentials, but also to reduce the number of school years from thirteen to twelve. (Such a proposal seems unlikely to gain acceptance in all of West Germany, though individual *Länder* may accept it.) In a recent speech, Riesenhuber disputed claims that 25-year-old university graduates left to do their own research might be 'irreverent'. "Irreverence in thinking is of significant value to science", he said.

Riesenhuber admits that arriving at European agreements on ethical matters such as research on human embryos or manipulation of the human germ line would be "extraordinarily difficult", but claims the situation is "not hopeless". In Riesenhuber's view, it is tolerable that European states should differ on complex matters as long as there is agreement on fundamental ethical questions.

Although he does not expect a unified set of European norms for such research in the EC, Riesenhuber said it is desirable that the various points of view "approach each other" as a result of public discussion involving researchers in the natural sciences, philosophy, theology and the law. "Parallel political decisions will only be possible" if such experts can arrive at some sort of consensus in advance. Experience in West Germany shows that the groups have not differed nearly as much as was expected.

Steven Dickman

Science board one answer to NRC's mounting problems

Washington

THE US Nuclear Regulatory Commission (NRC) seems to have a unique ability to infuriate its critics. The latest brouhaha came last month when it granted permission to the Tennessee Valley Authority (TVA) to restart the Sequoyah 2 nuclear power plant, closed since 1985 because of questions about its safety performance.

The outcry that followed might have been predicted. At the time of the decision, NRC was still investigating Steven White, TVA Manager of Nuclear Power, for lying to officials about safety matters at Sequoyah 2 and other nuclear plants. For its critics, this was further proof that NRC was incapable of making reasonable decisions.

There is no shortage of examples of regulatory problems with which NRC must contend. A report by the Critical Mass Energy Project — a public interest organization started by Ralph Nader — says that by March 1988 NRC had received reports of some 2,810 'mishaps' at nuclear power plants in 1987. Kenneth Boley, one of the report's authors, gives examples of NRC's failure to conduct thorough safety reviews are legion and that a particularly egregious example is the South Texas Nuclear Project. He claims NRC reviewed only a few of the 400 safety allegations brought against the South Texas Nuclear Project before granting it an operating licence last month.

A report of a subcommittee of the House of Representatives Committee on Interior and Insular Affairs is at least as critical. The report, *NRC Coziness with Industry*, argues that the agency fails to keep an "arms-length regulatory posture with the commercial nuclear power industry".

But Carl Goldstein of the US Committee on Energy Awareness, an industry lobby group, says NRC is an effective regulator, capable of taking tough action when necessary. If anything, the industry has been critical of NRC for being overzealous in its regulatory actions. But he agrees that the entire regulatory system needs changing, and NRC is coping the best it can in the circumstances.

NRC suffers from organizational problems that detract from its effectiveness. A five-member commission must approve all decisions, adding time to the decision-making process. Lando Zech, the present chairman, agrees that the current system is unwieldy, but he quickly adds that NRC "is doing an excellent job of assuring protection of public health and safety within the current structure".

For years there have been calls to replace the commission with a single administrator, and now they seem to be

heard. The House Interior Committee is holding hearings on legislation that would create a single NRC administrator, and similar legislation has been introduced in the Senate.

But there are political considerations that may scuttle any legislation this year. Democrats are loath to pass a bill that would give a lame-duck President Reagan the authority to appoint an administrator. Republicans fear that a Democratic president might install an administrator unfriendly to the nuclear industry. Certainly Massachusetts Governor Michael Dukakis, front-runner in the race for the Democratic presidential nomination, has not been cordial towards the nuclear power plants providing electricity for his state.

A 3-2 majority, including Zech, in the current commission favours a move to a single administrator. But Zech is not enthusiastic about congressional proposals for an independent science board to review NRC's actions. He would favour a



TVS's Sequoyah 2 nuclear plant back in operation but questions persist.

science board that reported to a single administrator, but rejects an independent board that would have authority to make decisions but would not take responsibility for them.

Supporters of an independent science board say it would be a valuable asset to NRC. Physics professor Harold Lewis of the University of California at Santa Barbara feels that a strong independent board could help NRC take difficult decisions. NRC already has an Advisory Committee on Reactor Safety, but Lewis, a member of that body, says it has been too narrowly focused on technical issues to provide the kind of thorough scientific overview that is needed.

Congressional support for an independent science board is only luke-warm. The Department of Energy has established an independent committee to watch over the activities of its nuclear reactors, and the fortunes of that body will no doubt influence opinion about a similar body for NRC.

Joseph Palca

Magnox costs trouble CEGB

Bristol

THE future of Britain's eleven Magnox nuclear power stations seems increasingly in doubt following stringent safety requirements imposed on two of the oldest stations as part of a condition of their continuing operation beyond their expected safe operating lives. The Nuclear Installations Inspectorate (NII), a branch of the state-financed Health and Safety Executive, has published its findings in a long-term safety review of the Magnox power station at Berkeley, near Bristol. Last July, a similar report was prepared for the Bradwell Magnox station in Essex.

A Magnox reactor is gas-cooled and fuelled by uranium bars contained within magnesium alloy ('Magnox') cans, with graphite moderator. The reactor core is mounted within a cylindrical mild-steel pressure vessel housed in a concrete biological shield of minimum thickness 8 feet. The Berkeley station has two reactors, each capable of generating 137.5 MW of electricity. Both Bradwell and Berkeley were commissioned in 1962, with expected useful lives of 20 to 25 years.

When the state-owned Central Electricity Generating Board (CEGB) indicated that it wished to continue to operate the stations beyond their originally estimated lifespan, it agreed to review the safety of each station after 20 years. In its report on Bradwell, the NII accepted that no immediate danger was presented by the continued operation of the reactors, but listed 17 key requirements that the CEGB would have to meet in order to be allowed to run the station until 1992. The verdict on Berkeley was similar, and the cost to the CEGB of fulfilling the requirements is estimated at £4 million per station.

Berkeley is at present operating at reduced pressure levels because of defects in welds in parts of the ducts that carry the coolant. Although the NII is satisfied that the ductwork is safe for another two years, it wants the CEGB to demonstrate the ductwork's integrity until 1992 by ultrasonic data. The CEGB, whose chairman Lord Marshall has likened the NII to a "nagging wife", has yet to decide whether the exercise is economically worthwhile.

With the issue further complicated by the impending privatization of the electricity supply industry and the fact that the Sizewell B pressurized-water reactor station is scheduled for completion by 1994, when it will be able to produce a relatively huge 1,175 MW, the CEGB is expected to let the NII know within the next few months what it intends to do about the two Magnox stations. Their closure seems a distinct possibility.

Simon Hadlington

Will industry bail out the Australian education system?

Sydney

A SPIRITED public debate about the financing of higher education in Australia has been triggered by the estimates in the federal government's green paper (consultative document) that increases in student enrolment by the end of the century will result in a A\$1,000–2,000 million deficit in tertiary education budgets.

Acknowledging the potential problem, the Minister for Employment, Education and Training, Mr John Dawkins, has convened a committee headed by a former New South Wales Premier, Mr Neville Wran, to investigate alternative ways of raising extra funds. But the various groups on whom the financial burden is likely to fall are making their views heard in advance of the committee's recommendations to the government, due on 3 May.

One option is to make students responsible. Wran has ruled out the reintroduction of tertiary fees on the pre-1974 pattern, but academic and student groups have already mounted a campaign against any form of fees system.

Another option is a tax deducted from the wages of graduates above some

income threshold until education costs are recouped, but there are difficulties. Geoffrey Lehmann, a senior national tax consultant, says that the "imposition of a graduate tax would result in massive tax avoidance and an exodus of young graduates from Australia".

According to union and student groups, a better course of action would be to seek extra financial support from industry, as corporations are the main beneficiaries of graduates leaving the tertiary education system. There are two proposals for increased participation from business. One calls for a 2 per cent tax on company revenues, providing a return of A\$330 million. Or employer contributions might take the form of a fund to provide money for student training schemes.

But employer groups are strongly opposed to such mandatory programmes. George Hutton, assistant director of the Business Council of Australia, says that "further taxes and levies" would reduce the ability of the business communities to employ graduates and that it is for individual companies to decide how their money is spent.

Tania Ewing

Bennett and Stanford tangle on restructured curriculum

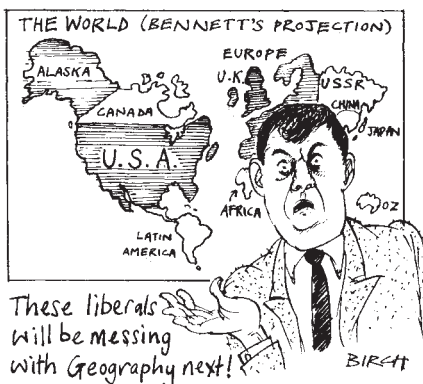
Berkeley

US SECRETARY of Education William Bennett let loose a verbal barrage against Stanford University last week in his campaign for a return to traditional standards for US undergraduate education. He accused Stanford of "trashing Western culture" by restructuring its core course on Western culture to include issues of gender, race and class, and elements of non-Western culture. Bennett says Stanford's decision was political, not educational and that it represents a "capitulation to a campaign of pressure politics and intimidation" by students.

Bennett fears that where Stanford leads others will follow. His most recent criticisms came in a speech on 18 April at Stanford itself, followed by a televised exchange with Stanford president Donald Kennedy. Kennedy accused Bennett of using "the privilege of his pulpit to bully rather than to engage the issues". Stanford faculty on both sides of the issue have expressed surprise at Bennett's meddling in Stanford's affairs, and say he is misinformed about the process that shaped the curriculum change.

Stanford had required a one-year course in Western civilization of all entering students. Freshmen choose from eight

options each with a unique focus, such as 'Great Works' or 'Values, Technology, Science and Society', but all bound together by a required reading list of 15



These liberals
will be messing
with Geography next!

classic Western works, to which faculty members may make their own additions.

The core reading list has been criticized by both students and faculty for not sufficiently representing the contributions of women and non-Western cultures. This led, in 1986, to the appointment of a task force to examine and redesign the course.

After two years of debate, at least one work from a non-Western culture and three works dealing with issues of gender, class or race will be incorporated in each

Lords fight for academic freedom

London

THE British government's proposals to reform the financing and management of higher education continue to run into fierce opposition, despite an earlier promise of several concessions (see *Nature* 331, 645; 1988). Last week it was the turn of peers, when the Education Reform Bill had its second-reading debate in the House of Lords. Attention focused on the question of the protection of intellectual freedom following the abolition of the universities' right to offer tenure to academic staff.

Lord Jenkins, chancellor of the University of Oxford and one of the country's most distinguished parliamentarians, said the bill as it stood made him "extremely doubtful whether we will have a university of foremost world class in the country by the end of the century". The Lord Chancellor, Lord Mackay, insisted that the government had not "closed its ears or minds" to suggestions to include a specific reference to academic freedom in the bill. The matter would be reconsidered at the committee stage. He reminded peers that the government had already stated its intention to include amendments to set up grievance procedures that would ensure the protection of intellectual freedom.

Before the debate, a newly formed pressure group, the Committee of University Autonomy, presented the Lords with a petition carrying the signatures of 6,000 academics protesting against the "current threat to our universities and colleges and to their intellectual liberties". The committee says that a university's ability to offer tenure is an important selling point in an increasingly competitive international market place and that if the government refused specifically to protect academic freedom, more staff would seek secure positions abroad.

Simon Hadlington

option. The core list of Western works has not been abandoned, but will be revised each year. Next year's list includes the Bible and works by Machiavelli, Rousseau, Plato, St Augustine and Marx.

Bennett has criticized Stanford administrators for basing their decision not on "enlightened debate" but on "tactics of intimidation" by student factions. Those involved seem to disagree. Economics professor Kenneth Arrow, an opponent of the new curriculum, called the motives for change "responsible and thoughtful" and denied that faculty senate members had responded to student pressure. The faculty senate voted 39 to 4 on 31 March to approve a compromise that retains a core list but allows greater freedom for incorporating other works, including those dealing with the newly designated issues.

Marcia Barinaga

Psychiatry in the Soviet Union

SIR—Several points in S.P. Calloway's uncritical defence of the late Professor Aleksandr Snezhnevskii as "a leading and highly respected Soviet psychiatrist" who "never described political dissent as a form of 'creeping schizophrenia'" (*Nature* 331, 296; 1988) require comment.

Although Snezhnevskii was for many years head of the Institute of Psychiatry in Moscow, he had been director of the Serbskii Institute of Forensic Psychiatry — notorious for its close links with the KGB¹ — in the 1950s². During this period he developed his theories of mental illness, particularly his broad concept of schizophrenia, which have virtually dominated Soviet psychiatric thinking over the past 30 years.

The ascendancy of Snezhnevskii's theories and diagnostic system led to a stretching of the boundaries of mental illness. His classification of schizophrenia postulates several forms and subtypes, the mildest labelled sluggish ("creeping") schizophrenia, which allows the most subtle behavioural changes to be interpreted as evidence of a severe psychiatric disorder³. As a result, dissidents were readily characterized as having, for example, "reformist delusions"⁴ and were incarcerated in psychiatric institutions^{3,5}. Western criticisms of the political abuse of psychiatry in the Soviet Union were repeatedly repudiated by Snezhnevskii as anti-Soviet slander, charges which the authorities have since admitted to, albeit grudgingly⁶.

Snezhnevskii not only defended Soviet psychiatry in international forums, but, *pace* Calloway, himself participated in the psychiatric internment of dissidents^{3,5}. After a careful consideration of the evidence, the Committee on Abuse of the Royal College of Psychiatrists concluded in 1979 that he had "acted unethically" and that "his direct involvement in the misuse of Soviet psychiatry" was "incompatible" with the privilege of honorary membership of the college³. Rather than defend himself against the charges, Snezhnevskii resigned from the college, a move that the All-Union Society of Neurologists and Psychiatrists of the USSR was to follow in 1983 when it faced expulsion from the World Psychiatric Association.

Leading German psychiatrists played a fundamental role in the Nazi programmes of mass sterilization and murder of psychiatric patients as well as the extermination of other "undesirables"⁷. After the Second World War, Carl Gustav Jung urged colleagues to raise the alarm if the abuse of psychiatry ever recurred, warning that silence would be tantamount to complicity⁸. The opposition to Snezhnevskii and his accomplices originated with Soviet psychiatrists themselves, some of

whom have suffered dismissal (Kazanetz), imprisonment (Gluzman, Koryagin) and exile (Voikhanskaja, Voloshanovich) as a result. Dialogue between Western psychiatrists and the heirs of Snezhnevskii is futile and disgraceful if the former remain silent when faced with a perversion of their own professional ethics and standards.

KAREL W. DE PAUW

Psychiatric Department,
Leicester General Hospital,
Gwendolen Road,
Leicester LE5 4PW, UK

1. Lader, M. *Psychiatry on Trial* (Penguin, London, 1977).
2. Reich, W. *Archs gen. Psychiat.* 36, 1029–1030 (1979).
3. Bloch, S. & Reddaway, P. *Soviet Psychiatric Abuse: The Shadow over World Psychiatry* (Gollancz, London, 1984).
4. Merskey, H. & Shafan, B. *Br. J. of Psychiat.* 148, 247–256 (1986).
5. Medvedev, Z. & Medvedev, R. *A Question of Madness* (Macmillan, London, 1971).
6. Rich, V. *Nature* 330, 5 (1987).
7. Müller-Hill, B. *Tödliche Wissenschaft: die Aussonderung von Juden, Zigeunern und Geisteskranken 1933–1945* (Rowohlt, Reinbek bei Hamburg, 1984).
8. van Voren, R. *Tijdschr. Psychiat.* 25, 67–69 (1983).

Earth science review

SIR—Your leading article¹ on the progress of the Earth Science Review is misleading. To say that Oxburgh "advocated open recognition" that some geology departments had "no continuing claim on public, let alone academic, attention" is a misrepresentation of the conclusions of that report. Furthermore, the clear implication of your statement is that those departments that the University Grants Committee (UGC) has now chosen to close, or to run down, were worthless departments.

In the first place, we know of no UK geology/Earth science department that deserves the description unworthy of academic attention. We accept, indeed welcome, the principles of rationalization and concentration of resources in fewer departments, but it must be clearly understood that this does not indicate the existence of a substantial number of moribund departments. Many departments may have a measure of dead wood, but it is to be found as much in the departments now favoured by the UGC as in those not supported. Certainly some departments were stronger than others. However, a recent UGC analysis² of research excellence in university departments shows a pattern in the Earth sciences that bears little relationship in part to the list of departments that the UGC now intends to expand and support. Three departments then regarded as 'above average' are to be effectively closed by the UGC, while six 'average' departments and one 'below-average' department are to be retained. At the time, there was some argument about the criteria used in the review, but that is not the point at issue here. What it

does show quite clearly is that the presently proposed closure of a department does not mean that it was unworthy of support in the sense that your leading article implied. It is obvious that academic performance was not the only criterion, and in some cases not even an important criterion, underlying the results of the Earth science review.

B. A. O. RANDALL
J. M. JONES

Department of Geology,
The University,
Newcastle upon Tyne NE1 7RU, UK

1. *Nature* 332, 2 (1988).
2. UGC letter to University Vice-Chancellors Planning for the late 1980s (May 1986).

Insurance and AIDS

SIR—Your timely leading article (*Nature* 331, 288; 1988) points out some of the problems that insurers will face with the spread of AIDS. Impertinent questions and unreliable blood tests are not an acceptable means of dealing with these problems in a civilized society; moreover, they are ineffective. Levels of risk among different groups will change as the disease develops; and even if a blood test produces a correct result, the applicant can go out and get infected the next day.

But you did not mention another approach, which seems to offer a reasonable solution to most of the problems of AIDS and life insurance at rather less cost. Most life policies do not exclude suicide; and suicide is more voluntary, faster and more dangerous than AIDS. There is, of course, *some* protection for insurers: normally, they do not pay out on a suicide if the policy is less than a year old.

If AIDS-related death were considered as a sort of slow suicide — with, say, a five-year rather than a one-year exclusion — then insurers would have at least as much protection as they could hope to gain through blood tests, but at rather less expense; and privacy would not need to be invaded.

MARTIN KOCHANSKI

7 Courtfield Gardens,
London SW5 0PA, UK

Creationism

SIR—A few more questions on creationism (*Nature* 331, 10; 1988). If God created life, then who created God? And if God was created by Himself why could not life do the same?

ALDAR S. BOURINBAIAR
PATRICIA G. MCCAFFREY
EDDIE SCHINDLER
NORIKO HIRAYAMA

Happy Heights 207,
Kurata,
703 Okayama,
Japan

Explosion fragments by numbers

What happens when an explosive shell blows up, or when an array of galaxies is formed in a "big bang"? There will be fragments, but calculating their properties is a complicated matter.

EXPLODING shells kill people unfortunate enough to be nearby mostly by the impact of fragments of their casings, which is why shell-designers must arrange that there are as many lethal fragments as possible. Plainly, there are complicated trade-offs to make. To kill a person, a fragment must carry enough momentum to cause lethal damage, to a first approximation just that momentum needed to punch a hole in the human body. But momentum in excess of that threshold is a waste of kinetic energy.

So the optimum solution (if that is the phrase) is that in which there are as many fragments as possible with threshold momentum. The snag is that the total kinetic energy to send the fragments flying is the explosive power of the shell less whatever energy is spent in the fragmentation of the case: more fragments mean that less kinetic energy is available.

The military have been occupied with these recondite questions at least since early in the Second World War. So much is clear from the list of references for an article by Brad Lee Holian from the Los Alamos National Laboratory and Dennis E. Grady from the Sandia National Laboratories, whose earliest reference is to an unpublished British Ministry of Supply report in 1943 by N.F. Mott (now Sir Nevill Mott) and E.H. Linfoot (*Phys. Rev. Lett.* **60**, 1355; 1988).

Mott explains that part of his wartime work in what was then called operations research required a relationship between lethality and fragment size, and that curiosity impelled him and Linfoot to look into the expected distribution of fragments with size. That is the aspect of this distant but not forgotten work to which Holian and Grady refer.

Luckily, there is more to be said about fragmentation than the lethality of exploding shells. Holian and Grady also refer to the big bang with which the Universe is supposed to have begun, and show that their calculations have something to say about the present distribution of galaxies with mass. But there are many other applications, while their technique is at once novel and potentially important. It may also encourage people by suggesting that there is still hope of an analytical treatment of the problem.

The essentials of the technique are borrowed from molecular dynamics and are applied, for the sake of making the problem tractable, to a two-dimensional structure in which elements of the material

(think of them as atoms) are enclosed in a square piece of a square lattice. The particular calculation described rests on the assumption that elements of the fragmenting material interact with their nearest neighbours only by means of a Lennard-Jones potential — the difference between a repulsive inverse sixth-power potential and an attractive inverse twelfth-power potential. By imposing periodic boundary conditions, the square piece of the square lattice can represent an infinite two-dimensional sheet. To obtain worthwhile statistics, it is necessary to put as many as 4,200 elements in each expanding square.

To make the molecular dynamics work, Holian and Grady simply set the lattice expanding isotropically, so that the initial relative velocity of an arbitrary particle is a linear function of its distance from the point of reference, but the computer codes arrange that, if a particle leaves the basic lattice block by crossing one edge, another will enter with the same velocity at the corresponding point of the opposite edge. The particles originally have a certain amount of energy, or a non-zero temperature. Once the system has been set moving, the particles simply obey Newton's laws, which is to say that the fragmentation is adiabatic.

Qualitatively, the results are as neat as one could hope. The first stages of the expansion entail merely that the underlying chequerboard structure stretches as atoms are pulled apart and thus, on the average, away from their equilibrium positions in the interaction potential, which implies a reduction of temperature. But that cannot last. Eventually, irregular holes appear in the fabric of the material and, because the model simulates surface tension, grow (allowing the temperature modestly to increase as the potential energy in nearest-neighbour linkages stretched almost to breaking is converted back into kinetic energy). Finally, as the simulation continues, the outcome is a collection of clusters ('fragments') of varying size and shape so irregular that their definition is hardly child's play.

What matters is whether the outcome is realistic. The simulations (five have been carried through for the purposes of this article) show that the most common fragments are small. In one computer run (nobody says how long it took, or on what kind of machine), the largest cluster contains 90 atoms, but there appear to have been 600 clusters in all, most of them

containing just two or three (or even one) atoms.

The logarithm of the number of clusters with more than a specified number of atoms is a decreasing linear function of that number, but there is a sharp change of slope of the curve that argues for a bimodal distribution such that the number of clusters larger than M is of the form $a^{-M/\mu} + b^{-M/\nu}$, where a , b , μ and ν are constants.

This, according to Holian and Grady, agrees with such experiments as there are. First, there are data gathered by setting off explosive charges in heavy steel cylinders and measuring the sizes of the fragments found and there is also the case of the Universe, where again the logarithm of the number of galaxies whose absolute luminosity exceeds M is a decreasing linear function of M (with no sign of a small galaxy tail in this case).

The authors say that their result conflicts with the old calculation of Mott and Linfoot, who concluded that the distribution would be exponential with diameter. But it is too early to be so sure. There remain gaps in Holian and Grady's argument, not the least of which is that their five simulations all correspond to starting-conditions in which there is enough kinetic energy to make the simulated materials liquid. Solids (attainable even in two dimensions with the Lennard-Jones potential) might be different.

That is not to complain. Grady, from Sandia, five years ago used macroscopic energetic considerations to calculate the average mass of fragments on the explosive impulse. It is largely a matter of telling what proportion of the initial kinetic energy will go into the creation of free fragment surface, from which it emerges that average fragment size decreases as the original impulse increases. But the simulations, when the parameters are suitably scaled, yield average sizes smaller by at least an order of magnitude. The explanation, interesting if not surprising, is that the simulated fragments are far from equilibrium (too much surface has been created).

It would be pleasing if the next step were a full three-dimensional simulation. Ordinarily, one might expect the military to be willing to support the most elaborate studies bearing on the way weapons work. Is it possible that they have become smart, and understand that shell casings are really two-dimensional? **John Maddox**

Heat-shock proteins

Coming in from the cold

Hugh Pelham

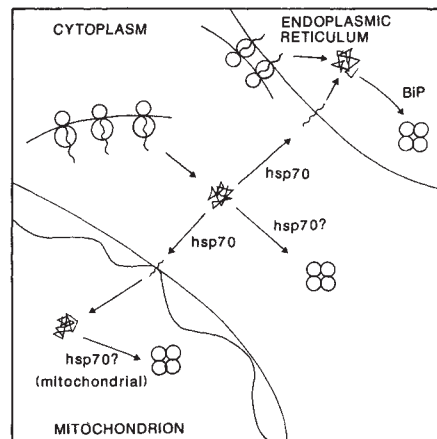
Most papers about heat-shock state in their introductory paragraph that heat-shock proteins are produced when cells are stressed, that they are highly evolutionarily conserved, that they, or closely related proteins, are also present in unstressed cells, and that their function is unknown. Two papers on pages 800 and 805 of this issue^{1,2} seriously threaten the simplicity of this last statement, because they provide, for the first time, a biochemical assay for a function of the major heat-shock protein, hsp70, that is of proven physiological significance.

Evidence for various activities attributable to members of the hsp70 family has been accumulating for several years, although the physiological relevance of these activities under normal growth conditions has always been disputable (see ref. 3 for review). The activities include ATP-dependent disruption of the clathrin coat of coated vesicles; the catalysis (by the *Escherichia coli* homologue *dnaK*) of a step in the initiation of phage lambda DNA synthesis; a role in the recovery of nucleoli following heat-induced damage; and ATP-reversible binding to several proteins including the transformation-related protein p53 and (for the hsp70-like protein BiP) various abnormal or incompletely assembled proteins in the endoplasmic reticulum. On the basis of such evidence, we suggested^{3,4} as a general model that hsp70 binds to hydrophobic regions of proteins, either naturally exposed or revealed as a result of denaturation, that binding can be reversed with the aid of ATP hydrolysis, and that it results in prevention or disruption of inappropriate protein-protein interactions.

Studies on the trans-membrane translocation of newly synthesized secretory and organellar proteins show that such proteins have to be maintained in an unfolded state to cross the membrane^{5,6}. Moreover, post-translational transport of some mitochondrial and secretory proteins requires both cytosolic factor(s) and ATP hydrolysis⁶, and it is possible⁷ that this requirement reflects the need for an ATP-dependent 'unfoldase' to create and maintain a loosely folded configuration of the precursor proteins. Such a role is strikingly similar to that proposed for hsp70, and it seemed logical to test whether the latter was involved in translocation.

The work reported in both papers in this issue shows that hsp70 is indeed one of the cytosolic factors involved in the translocation of proteins across membranes. Chirico *et al.*² used uptake of yeast prepro- α -factor (synthesized in a wheat-germ

extract) into yeast microsomal vesicles as an *in vitro* assay for translocation. They purified an activity from a yeast post-ribosomal supernatant that stimulates uptake into the microsomes. The purified material contains two proteins of relative molecular mass 70,000 (70K) which, by immunological criteria, are related to



Schematic drawing of proteins being synthesized by free or membrane-bound ribosomes, folding incorrectly and then being helped to reach membranes (which they cross in the unfolded state) or to fold into a final form by members of the hsp70 family in the cytosol, endoplasmic reticulum and mitochondrion.

hsp70. Two-dimensional gel analysis confirms that these proteins correspond to the products of the *SSA1* and *SSA2* genes, the two hsp70 family members (out of nine cloned so far) that contribute most of the cytosolic hsp70 in unstressed yeast cells. The authors therefore suggest that hsp70 is responsible for ATP-dependent unfolding or disaggregation of the prepro- α -factor before translocation.

A direct demonstration that the unfolding step is rate-limiting comes from experiments in which the substrate is denatured with urea before dilution into the translocation assay: such treatment increases the rate of translocation more than tenfold. The present data do not prove, however, that hsp70 interacts directly with the precursor, nor that ATP hydrolysis catalysed by hsp70 is essential for the effect. There may be other ATP-requiring steps in the import process, and at least one other soluble factor appears to be involved².

All these results were obtained using a somewhat artificial *in vitro* assay, but Deshaies *et al.*¹ in the other paper in this issue exploit the genetics of the yeast system to test the role of hsp70 *in vivo*. Disruption of three different hsp70 genes (*SSA1*, *SSA2* and *SSA4*) is sufficient to lower the

cytosolic level of hsp70-like proteins. Strains carrying the triple deletion are non-viable, but can be rescued by plasmids which express a single hsp70 protein under the control of the *GALI* promoter¹. Deshaies *et al.* grew such a strain in the presence of galactose, then added glucose to repress the *GALI* promoter and examined the effect of hsp70 depletion on transmembrane protein transport.

They find that not only does untranslocated prepro- α -factor accumulate in the cells, but so also does the unprocessed precursor of the β -subunit of the mitochondrial F_1 ATPase, implying that hsp70 is involved *in vivo* in importing proteins both into the endoplasmic reticulum and into mitochondria. A lesser effect is seen with the vacuolar enzyme carboxypeptidase Y and the secreted enzyme invertase, probably because these proteins are inserted into the endoplasmic reticulum membrane mostly co-translationally and thus have no need for an 'unfoldase'. The authors go on to show that purified hsp70 is active in prepro- α -factor translocation *in vitro*, and that cytosol from the hsp70-deleted strain (after glucose repression) is correspondingly deficient in such activity.

These results suggest that one function of hsp70 is to protect precursor proteins until they reach the site of membrane insertion. But what happens when the unfolded proteins emerge into their new environment? The endoplasmic reticulum contains another hsp70 family member, called BiP (also termed GRP78)³, and at a recent meeting at Cold Spring Harbor, Elizabeth Craig, co-author of the paper in this issue¹, reported that the product of one of the yeast hsp70 genes is transported into mitochondria. Thus, the emerging proteins in each organelle can once more be substrates for the attention of hsp70-like proteins.

BiP is known to associate transiently, and in an ATP-reversible manner, with immunoglobulin heavy chains³. It also binds to several mutant or abnormal proteins *in vivo*. Such interactions also occur when proteins synthesized *in vitro* enter dog pancreatic microsomes⁸. By manipulating the conditions of synthesis, Kassenbrock *et al.* show⁸ that yeast invertase binds to BiP if its glycosylation is blocked, but not if the normally glycosylated protein is produced. Furthermore, BiP binds to reduced or incorrectly disulphide-bonded prolactin, but not to the correctly bonded species. Thus, BiP interacts preferentially with incorrectly folded proteins, but does not bind newly synthesized proteins if they have achieved their mature folded state.

How exactly do BiP and the other members of the hsp70 family of proteins function? One can imagine these proteins seek out incorrectly or incompletely folded proteins, protect them from aggregating with each other (a 'chaperoning'

role, as discussed previously^{3,9}), and perhaps also unfold them a little using the energy of ATP hydrolysis³, as originally suggested for the putative unfoldase⁷. In this way, proteins that had reached a kinetic dead end in the folding pathway could be helped out of their predicament⁷. For mature polypeptides in the cytosol, endoplasmic reticulum or mitochondrion, this process would continue until the protein assumes its final configuration, at which point it would no longer be a substrate (see figure). Precursor proteins would be inhibited by their signal or import sequences from attaining a tightly folded structure in the cytoplasm, and so would be continually maintained in a loose configuration until they had found the appropriate membrane receptor. Only in the case of an abnormal or mutant protein would binding to an hsp70-like protein be a permanent fate.

The functions of the hsp70 family could be complemented by those of other proteins, particularly in the case of assembly of multi-subunit structures⁹. For example, chloroplasts of higher plants contain polypeptides of about 60K which bind to newly synthesized large and small subunits of the photosynthetic enzyme Rubisco and are required for the orderly assembly of this enzyme⁹.

Hemmingsen *et al.* now show¹⁰ that these binding proteins are related at the amino-acid sequence level, as well as in their biochemical properties, to the groEL protein, a major heat-shock protein of *E. coli*. One known function of the groE protein is to catalyse the assembly of the head of bacteriophage T4, which it appears to do by binding to the unassembled form of the major head protein and preventing the formation of amorphous aggregates, a 'chaperoning' role similar to that proposed for hsp70. Like the dnaK protein (the *E. coli* equivalent of hsp70), groE has weak ATPase activity. Proteins related to groEL (termed chaperonins by Hemmingsen *et al.*¹⁰) have been identified in several prokaryotes, as well as in chloroplasts and mitochondria, but not so far in the endoplasmic reticulum or cytosol of eukaryotes. They may well turn out to be as widespread and important as the hsp70 family.

What conclusions can be drawn from these findings? First, the results lend support to the idea³ that protein folding and assembly, long considered to be spontaneous processes, are actively catalysed *in vivo* in every cellular compartment in which they occur. This may be of consider-

able interest to those currently struggling with the task of reconstituting a complex protein from the insoluble material obtained from its overexpression in a bacterial system, although it must be stressed that no magic cocktail of heat-shock or other proteins has yet been shown to ease this task. Second, the naive view that heat denatures proteins and that the role of at least some of the heat-shock proteins is to help to renature them may

not be so far off the mark. Certainly, the work reported in this issue^{1,2} suggests that for hsp70, at least, the era of speculation is drawing to a close, and the detailed biochemical analysis of an important and presumably widespread function of the protein can now begin. □

Hugh Pelham is at the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, UK, currently on leave at the Institute of Molecular Biology II, University of Zurich, Switzerland.

DNA biochemistry

G-strings at chromosome ends

Thomas R. Cech

TEN years ago the ends of the linear DNA molecules containing the ribosomal RNA genes of the protist *Tetrahymena thermophila* were reported¹ to consist of tandem repeats of the sequence $(C_4A_2) \cdot (T_2G_4)_n$. Since then, many eukaryotic telomeres have been sequenced, and all fit the same general pattern: a short DNA sequence, one strand G-rich and one C-rich, is tandemly repeated at the chromosome ends. Two recent papers^{2,3} provide important insight as to how these repeats are synthesized and how they might function in chromosome maintenance.

Greider and Blackburn² partially purify and characterize the terminal deoxynucleotidyltransferase from *Tetrahymena* that synthesizes the G-rich strand; they make the unexpected discovery that the enzyme has an essential RNA subunit. Meanwhile, Henderson *et al.*³ investigate the structure of the G-rich strands of several telomeric sequences, and find that they fold to form novel intramolecular structures containing G-G base pairs. In addition to its implications for telomere function, this work provides evidence for the biological relevance of a DNA structure not based on Watson-Crick G-C and A-T base pairs.

Unlike the circular chromosomes of bacteria, the chromosomes found in the nuclei of eukaryotes are linear DNA molecules. The ends of linear chromosomes pose a problem for DNA replication⁴. DNA polymerase normally extends an RNA primer; the gap left by removal of the primer is filled by extension of upstream sequences. At the end of a DNA molecule, however, there are no upstream sequences to extend. Telomeres, the natural ends of linear chromosomes, must have a mechanism to prevent shortening of the chromosome with successive rounds of DNA replication.

De novo synthesis of telomeric DNA repeats suggests one solution to the telomere replication problem. Artificial chromosomes can be maintained in yeast if they are terminated with *Tetrahymena* $(T_2G_4)_n$ or *Oxytricha* $(T_4G_4)_n$ telomeres; in

the process, additional telomeric repeats with the yeast $(TG_{1-3})_n$ sequence are added to the chromosome ends^{5,6}. These and other results pointed to the existence of a telomere terminal transferase (telomerase). Such an activity was subsequently identified in *Tetrahymena*⁷; it extends a $(T_2G_4)_n$ primer with more of the T_2G_4 sequence, and does not require an external template. One possible model for telomere replication using such a telomerase is shown in the figure.

Greider and Blackburn now announce² that telomerase is a ribonucleoprotein enzyme whose RNA and protein components are both essential for activity. The evidence for the RNA component includes the sensitivity of the activity to micrococcal nuclease and to ribonuclease A, and the copurification of specific small RNAs with the telomerase activity. What function might the RNA have? It could supply a C_4A_2 template to direct the synthesis of $(T_2G_4)_n$. Because RNA itself can have sequence-specific nucleotidyltransferase activity⁸, the RNA could provide both an internal template and the catalytic centre for polymerization. Finally, the RNA could be involved in primer recognition.

The telomerase from *Tetrahymena* does not elongate telomeres by adding preformed T_2G_4 oligonucleotides, but rather extends the chain one nucleotide at a time using dTTP and dGTP as substrates. The telomerase has specificity for certain single-stranded oligonucleotides as primers. For example, $(T_2G_4)_n$ ($n > 1$), $(T_4G_4)_n$ and $(TG_{1-3})_n$, the *Tetrahymena*, *Oxytricha* and yeast telomeric sequences, respectively, function as primers for synthesis of T_2G_4 repeats. A similar activity isolated from *Oxytricha* adds T_4G_4 repeats to $(T_4G_4)_n$ primers (A. Zahler and D. Prescott, personal communication).

In addition to its primer specificity, the *Tetrahymena* telomerase recognizes the sequence at the 3' end of the primer such that it fills out partial T_2G_4 repeats. So a primer ending in TGGG-OH is extended first with a G and then with two Ts,

- Deshaies, R. J. *et al.* *Nature* **332**, 800-805 (1988).
- Chirico, W. J., Waters, M. G. & Blobel, G. *Nature* **332**, 805-810 (1988).
- Pelham, H. R. B. *Cell* **46**, 959-961 (1986).
- Lewis, M. J. & Pelham, H. R. B. *EMBO J.* **4**, 3137 (1985).
- Zimmermann, R. & Meyer, D. I. *Trends biochem. Sci.* **11**, 512-515 (1986).
- Eilers, M. & Schatz, G. *Cell* **52**, 481-483 (1988).
- Rothman, J. E. & Kornberg, R. D. *Nature* **322**, 209 (1986).
- Kassenbrock, C. K. *et al.* *Nature* (in the press).
- Ellis, R. J. *Nature* **328**, 378-379 (1987).
- Hemmingsen, S. M. *et al.* *Nature* (in the press).

whereas one ending in TGGGG-OH is extended first with two Ts and then with four Gs. The activity is analogous to transfer (t)RNA nucleotidyltransferase⁹, which completes the 3'-terminus of a tRNA that is missing any portion of the CCA-OH sequence. There could even be an evolutionary relationship between telomerase and the tRNA CCA-adding enzyme. A tRNA-like structure with a 3'-terminal CCA could be the ancestral telomere for the RNA genomes that carried genetic information in the RNA world¹⁰.

An unusual DNA structure common to the telomere G-rich strands correlates with their ability to act as primers for telomerase. Henderson *et al.* apply³ the simple technique of polyacrylamide gel electrophoresis under nondenaturing conditions to synthetic oligonucleotides having G-rich telomere sequences. As the temperature is lowered from 37 to 19°C, conformational isomers with increased electrophoretic mobility are observed. At 5°C the shift to the faster-migrating species is highly efficient for some of the sequences. Short G-rich DNAs such as G₆, (TG)₅, and (GT)₅ show only minor changes in mobility as a function of temperature. These mobility shifts cannot be explained by intermolecular duplex formation or aggregation, and are consistent with the oligonucleotides folding in half to form hairpin stem-loop structures.

The structure of the putative intramolecular hairpins formed by the G-rich telomere strands is not known in detail, but nuclear magnetic resonance spectroscopy provides some clues³. The ¹H NMR spectrum of (T₂G₄)₄ at 9°C in aqueous solution shows about 24 imino proton resonances, more than could be provided by G-T base pairs in such an oligonucleotide. Therefore, G-G base pairs seem likely (see figure). Furthermore, two-dimensional NMR nuclear Overhauser effect experiments indicate that (T₂G₄)₄ contains 4-6 G residues in *syn* conformation (the base folded over the deoxyribose sugar instead of the *anti* conformation found in Watson-Crick base pairs). Thus, telomeric 'G-DNA' may contain *syn*G-*anti*G base pairs.

The evidence that G-DNA structure is important for telomere function is as yet only circumstantial: the ability of oligonucleotides to form the structure correlates with their ability to serve as primers

for telomerase. Thus, in addition to the challenge to physical chemists to solve the G-DNA structure, there is a challenge to biochemists to test the biological relevance of the structure. G-DNA at chromosome ends could have functions other than promoting telomerase, such as stabilizing the ends against degradation or recombination. The structure might be recognized by telomere-binding proteins³. Conversely, considering the very inf-

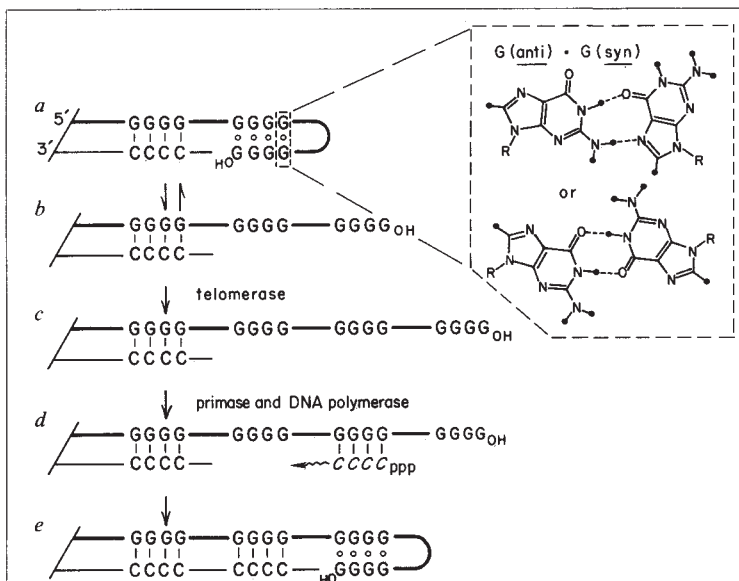
unknown, although it could organize different DNA molecules within the macronucleus. From a chemical point of view, it will be interesting to learn if the intermolecular coherence of *Oxytricha* telomeres involves the sort of G-G base-pairing found in the intramolecular hairpin formed by the G-rich strand of *Tetrahymena* telomeres.

The telomere DNA sequences described so far represent phylogenetically diverse

organisms including protozoa and fungi, but have not included higher animals or plants. A recent report by Richards and Ausubel¹³ indicates that the telomeres of higher eukaryotes are also composed of short tandem repeats of G-rich sequences. Using an imaginative cloning strategy to enrich for telomeric sequences, these authors cloned the telomeres of the flowering plant *Arabidopsis thaliana*, which turn out to be composed primarily of T₃AG₃ repeats. Their DNA hybridization studies indicate that a similar sequence is present at the telomeres of other plants, including the monocot *Zea mays* and various dicots, and at the telomeres of animals including humans^{13,14}.

It now seems that the telomeres of all eukaryotic nuclear chromosomes have fundamentally similar structures, and presumably a similar mechanism of replication. *Tetrahymena* and *Oxytricha*, with their abundance of telomeres and telomerase, provided the first information

about telomere structure and function at the molecular level. These ciliated protozoa will continue to have a key role in future studies of chromosome ends. □



Model for telomere DNA structure and mechanism of elongation. *a*, Telomeric DNA consists of tandem repeats of a short sequence, one strand G-rich and one C-rich. The G-rich strand (bold line) folds back on itself via G-G base-pairing (open circles). Two possible G-G base pairs are shown in the inset. *b*, The G-rich strand is unfolded and, *c*, additional repeats are added by the telomere terminal transferase. *d*, The C-rich strand can then be synthesized by RNA-primed replication (RNA shown in italics). *e*, Ligation of the C-rich strand, removal of the RNA primer and refolding of the G-rich strand complete elongation of the telomere. Alternatively, the folded G-rich strand could prime synthesis of the C-rich strand, although there is no experimental evidence for this. Telomere elongation is proposed to compensate for the shortening of the ends of chromosomes that is the consequence of normal DNA replication⁴.

ficient formation of the collapsed G-DNA structure by (T₄G₄)_n, the *Oxytricha* telomere sequence, proteins may be necessary to stabilize G-DNA in some cases.

A different type of novel DNA structure is formed by the telomeric DNA of the ciliated protozoan *Oxytricha*. *Oxytricha* telomeres consist of 2.5 repeats of the sequence (C₄A₄)-(T₄G₄) with an additional single-stranded (T₄G₄)₂ extension at the 3' end. In high concentrations of NaCl, these telomeres form specific intermolecular complexes¹¹. Oka and Thomas¹² have recently shown that both the G-rich and C-rich strands are required for coherence of the telomeres. Strikingly, they find that once the structure is formed in Na⁺ it is uniquely stabilized by K⁺. It is very unusual to find a nucleic-acid structure stabilized by a specific monovalent cation. Four-strand and three-strand models have been proposed to explain the coherence of two telomeres. The biological role of such an interaction is

1. Blackburn, E.H. & Gall, J.G. *J. molec. Biol.* **120**, 33-53 (1978).
2. Greider, C.W. & Blackburn, E.H. *Cell* **51**, 887 (1987).
3. Henderson, E., Hardin, C.C., Wolk, S.K., Tinoco, I., Jr & Blackburn, E.H. *Cell* **51**, 899-908 (1987).
4. Watson, J.D. *Nature new Biol.* **239**, 197-201 (1972).
5. Szostak, J.W. & Blackburn, E.H. *Cell* **29**, 245-255 (1982).
6. Pluta, A.F., Dani, G.M., Spear, B.B. & Zakian, V.A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1475-1479 (1984).
7. Greider, C.W. & Blackburn, E.H. *Cell* **43**, 405 (1985).
8. Westheimer, F.H. *Nature* **319**, 534 (1986).
9. Deutscher, M.P. *The Enzymes* **15**, 182-215 (1982).
10. Weiner, A.M. & Maizels, N. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7383-7387 (1987).
11. Lipps, H.J., Grussem, W. & Prescott, D.M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2495-2499 (1982).
12. Oka, Y. & Thomas, C.A., Jr. *Nucleic Acids Res.* **15**, 8877-8898 (1987).
13. Richards, E.J. & Ausubel, F.M. *Cell* **53**, 127-136 (1988).
14. Allshire, R.C. *et al. Nature* **332**, 656-659 (1988).

Thomas R. Cech is in the Department of Chemistry and Biochemistry, University of Colorado, Campus Box 215, Boulder, Colorado 80309-0215, USA.

Plate tectonics

A close look at parting plates

William B. F. Ryan

THERE was a buzz in the air at a 'show and tell' session of a recent meeting*. The occasion was the presentation of an extraordinarily rich collection of planetary images. The planet was our own, and the panorama was of the ocean floor, until now well hidden by many kilometres of sea water. The novelty was imagery from remote sensing using sound instead of light or radar. These sonar pictures were

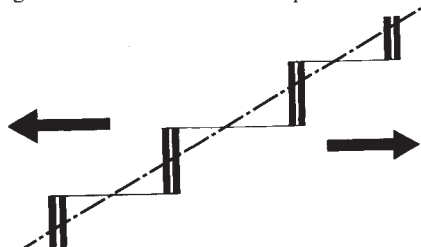


Fig. 1 The classical model of separating lithospheric plates. Spreading centres (bold stripes) driven and filled by rising magma, form perpendicular to the direction of plate motion (arrows) and are connected by transform faults (light lines). The stepped (*en echelon*) pattern lies along the mean direction of the plate boundaries (broken line).

pasted into mosaics that show an unparallel view of the young crest of the mid-ocean ridge in the Pacific Ocean. At a feature termed the East Pacific Rise, the ocean floor is seen to be steadily cracking in two because of the separation of the large lithospheric plates. Through the fissure molten rock from within the Earth's mantle is erupting to build the oceanic crust as elongated volcanic constructions.

A cause of controversy is the hypothesis that this accretionary plate boundary is segmented into adjacent but separate spreading cells, each driven magmatically but possibly by different plumes (Fig. 1). The scale of the segmentation is much debated. For several decades it has been established that the ridge was offset by sometimes very large transform faults across which the lithosphere slid by past itself with little extension or compression. Recently, however, sonar instrumentation towed near the sea floor for close inspection and near the sea surface for large-area mapping has revealed a much finer scale of ridge-axis discontinuities.

The best described and most hotly debated type of discontinuity is the overlapping spreading centre discovered by Peter Lonsdale and further explored by Ken Macdonald (University of California, Santa Barbara) and colleagues. One over-lapper 9° north on the East Pacific Rise (Fig. 2) is the subject of a detailed study

by Macdonald *et al.* in a recent issue of *Geology* (15, 993–997; 1987). A puzzling feature of this and all other overlapping spreading centres is the lack of transverse structures accommodating the extension of two spreading centres whose tips pass one another. Transverse slip is expected in classical plate-tectonic theory unless the overlappers are temporary phenomena. Macdonald *et al.* applied crack-propagation models to show that overlappers can be short lived and have a discernible evolution. The offsets seem to appear when discrete magmatic pulses originating in separate and segmented spreading centres misalign and run past each other. When this happens either one tip continues to propagate its tensional crack and eventually slices through to join with the adjacent *en echelon* ridge segment, chopping off the opposing tip, or it may cut inside or outside itself, repeatedly decapitating its own tip. The sonar imagery gathered in the spring of 1987 and on display for the first time at the meeting showed that overlapping spreading centres are commonly encountered down a 1,000-mile corridor straddling the axis of the East Pacific Rise.

The degree of magmatic segmentation within the individual spreading cells is also under scrutiny. Until last year, most geochemists imagined that there is a single magmatic source for each transform-fault-

bounded spreading cell. The magma is derived from upwelling mantle beneath the spreading centre that is drawn up by the separation of the plates. The rising material decompresses adiabatically and, when melting begins, magma segregates from the host rock, ascends and is then injected into the crust midway along the spreading cell. David Scott and David Stevenson (California Institute of Technology) presented numerical models used to investigate why volcanism at the ridge axis is confined to the very narrow belt imaged by the sonar devices. Their calculations show that the positive buoyancy of the low-density liquid magma in combination with the negative buoyancy of the residual solids relative to unmelted mantle is sufficient to focus a plume of ascending melt into a very narrow magma reservoir within the zone of crustal accretion.

The reservoir itself has now been imaged with reflection seismology, and corresponds to a low-velocity zone whose roof lies 1.5–2.0 kilometres beneath the summit graben of the ridge crest, a feature analogous to an extremely elongated caldera (see Fig. 2). The velocity structure indicates that only a small amount of partial melt might exist at any time in the axial magma chamber, where it is ponded on the top of the zone to produce a crisp reflection. Now, given the possibility of poor mixing of liquid melt in a predominantly mushy crustal reservoir, geochemists consider that magmatic segmentation occurs on scales even smaller than the overlapping-spreading-centre discontinuities. Submersible dives recently completed by Bill Bryan and Geoff Thompson (Woods Hole Oceanographic Institution)

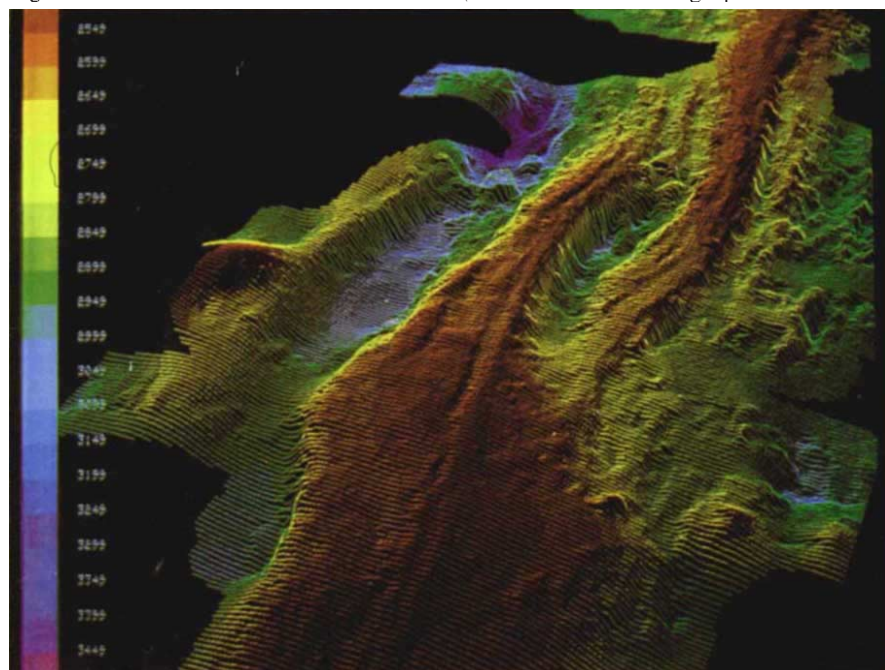


Fig. 2 Sonar image of the overlapping spreading centres 9° north on the East Pacific Rise (from the cover of *Geology*, November 1987; courtesy of K. Macdonald). The tips of the spreading centres extend past each other and there is no transform fault. The tips can grow to cut across each other. The slight dip at the top of the ridges is termed the summit graben. (Colour scale, metres depth.)

* American Geophysical Union fall meeting, 7–11 December 1987, San Francisco.

have sampled the lava terrain to determine the scale of along- and cross-axis variations in magma composition. The goal is to decipher the magmatic plumbing that feeds the ridge segments and the heterogeneity of the parent material.

The new sonar images hold out the prospect of deducing some key aspects of the spreading cells' magmatic plumbing directly from the cross-sectional shape of the ridge-crest topographic bulge. They hint that where the axial magma chamber is best developed, the high is broad and there is a summit graben. A shrinking of

the chamber along the axis away from the injection centre and towards the discontinuities seems to be mimicked by a tapering of the high into a more peaked ridge and the transformation of the graben into a swarm of fissures. Because the differences in morphology also appear off-axis on older crust, it is likely that sonar imagery will become useful for the study of changes in the magmatic budget and source locations back through time. □

William B. F. Ryan is at the Lamont-Doherty Geological Observatory, Palisades, New York 10964, USA.

Oxide superconductors

Clues from copper-free samples

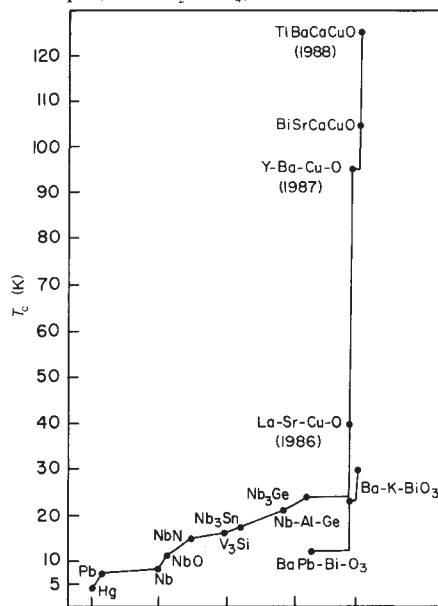
T. M. Rice

ALTHOUGH the superconducting transition temperature (T_c) of the compound $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ reported by Cava *et al.* on page 814 of this issue¹ is, at 30 K, much less than others reported over the past year (see figure), it is substantially higher than the highest value (23 K) obtained previously in conventional intermetallic compounds. The new system is closely related to one of the earliest superconducting oxides, $\text{BaPb}_{1-x}\text{Bi}_x\text{O}_3$ discovered by Sleight *et al.*² in 1975, for which the maximum T_c of 13 K occurs at $x \approx 0.75$. The discovery of this new family of copper-free oxide superconductors will help elucidate the characteristics necessary to achieve high transition temperatures.

After Bednorz and Müller's breakthrough³ in high-temperature superconductivity with the $\text{La}_{2-x}\text{Ba}_x\text{CuO}_4$ system in 1986, several groups attempted to make the analogous doping for the BaBiO_3 system. Instead of introducing carriers in the parent insulating compound by substituting on the active site (bismuth or copper) as was done with $\text{BaPb}_{1-x}\text{Bi}_x\text{O}_3$, they are introduced on the other site as in Bednorz and Müller's technique, thus keeping the bismuth (or copper) network intact.

The chemistry of substitution, however, is more difficult for the BaBiO_3 system. The first report of success came from Mattheiss *et al.*⁴ at AT&T Bell Laboratories who achieved a $T_c \approx 22$ K in a multi-phase sample of $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ but with a superconducting fraction of only 4 per cent. Cava, Batlogg and co-workers¹, also at AT&T, using a sealed-tube technique to contain the volatile potassium oxides, obtain single-phase material with $x \approx 0.4$ and a higher T_c (about 30 K). The superconducting fraction, measured as the volume from which magnetic flux is expelled (the Meissner effect), is greatly improved at 30 per cent. This new work establishes the bismuth oxides as the clear runner-up to the copper oxides in the high- T_c race.

It is naturally very interesting to compare and contrast the two oxides. There are several similarities. First, both are based on perovskite-type oxide structures. Second, both parent compounds are effectively single-valence insulators: for example, in La_2CuO_4 , each Cu^{2+} is one



Progress in superconducting transition temperatures (T_c) since Kamerlingh Onnes' initial discovery of superconductivity in 1910.

electron short of a 'filled' d^{10} -shell, giving one 'hole' per copper, and each Bi^{4+} in BaBiO_3 has an electron outside a filled shell. Third, superconductivity in both is achieved by introducing a few charge carriers by substituting ions, Sr^{2+} or Ba^{2+} for La^{3+} and K^+ or Rb^+ for Ba^{2+} , and superconductivity occurs in samples with stoichiometry close to the transition from insulating to metallic behaviour.

There are, however, noteworthy differences. The bismuth oxides are truly three-dimensional compounds in contrast to the two-dimensional or layered structure

of all the superconducting copper oxides. Also, the parent compounds are different types of insulators. The cuprate materials, La_2CuO_4 and $\text{YBa}_2\text{Cu}_3\text{O}_x$, are antiferromagnetically ordered 'Mott' insulators, the magnetic moment of neighbouring ions cancelling. But BaBiO_3 is diamagnetic, the bismuth ions having zero magnetic moment, and has a doubled unit cell corresponding⁵ to a formal charge ordering $\text{Ba}_2\text{Bi}^{3+}\text{Bi}^{5+}\text{O}_6$. Thus, in the cuprate materials, the superconductivity occurs as the antiferromagnetism is suppressed, whereas in the bismuth oxides, because Cava *et al.* report¹ a cubic perovskite structure for $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_3$, it seems that the unit-cell doubling associated with the charge ordering is suppressed.

This raises the question of whether the mechanism of superconductivity is similar in both systems. If the superconductivity in the Cu-O planes of the cuprates arises from an essentially magnetically induced interaction, then this mechanism is not simply transferable to the bismuth oxides. On the other hand, $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ seems a natural candidate for a 'bipolaronic' mechanism. In simple terms, the charge-ordered parent compound $\text{Ba}_2\text{Bi}^{3+}\text{Bi}^{5+}\text{O}_6$ can be regarded as a lattice ordering of bipolarons (or two-electron pairs) on alternate Bi^{5+} sites stabilized by the strong relaxation of the surrounding oxygen octahedra.

The effect of potassium-doping is to make these pairs mobile by changing the number of bipolarons per bismuth-site from the commensurate value of a half. This new system is the best candidate so far for the long-sought bipolaronic limit of superconductivity^{6,7}. Alternatively a common mechanism for the bismuth and copper oxides could be postulated if the superconductivity is a property mainly of the oxygen network. This can only be decided after detailed experiments on the normal and superconducting states.

A related set of questions concerns the possibility of raising T_c further in the bismuth oxides. Of possible dopant atoms, potassium seems optimal, because the similarity of the ionic radius of K^+ (1.33 Å) to that of Ba^{2+} (1.35 Å) should make it the least disruptive dopant. In the copper oxides, the really high transition temperatures (above 40 K) were achieved by more complex methods of doping by using quaternary compounds (see figure). Can something similar be achieved in the bismuth oxides? At present one can only speculate. □

1. Cava, R. J. *et al.* *Nature* **332**, 814–816 (1988).
2. Sleight, A. W., Gillson, J. J. & Bierstedt, P. E. *Solid St. Commun.* **17**, 27–28 (1975).
3. Bednorz, J. G. & Müller, K. A. *Z. Phys.* **B64**, 184 (1986).
4. Mattheiss, L. F. *et al.* *Phys. Rev.* **B37**, 3745–3746 (1988).
5. Cox, D. E. & Sleight, A. W. *Acta Crystallogr.* **B35**, 1 (1979).
6. Chakraverty, B. K. & Ranninger, J. *Phil. Mag.* **B52**, 669–678 (1988).
7. Prelovsek, P. *et al.* *J. Phys.* **C20**, 4229–4233 (1987).

T. M. Rice is at the Institut für Theoretische Physik, ETH-Hönggerberg, CH-8093 Zürich, Switzerland.

Liquid crystals

Numerical models of mesophases

C. C. Huang

SMECTIC (from the Greek for soap) is the name chosen for certain liquid phases with physical properties reminiscent of soap. Iridescent soap films, which show beautiful diffraction colours under a white light, do so mainly because the soap molecules form layered structures. Consequently, the characteristic feature of all smectic phases is their layered structure. It is generally believed that an attractive interaction between the molecules is essential for the formation of smectic phases. Using molecular-dynamic and constant-pressure 'Monte Carlo' simulations on a system of hard spherocylinders (cylinders capped at each end by a hemisphere of the same radius) however, D. Frenkel, H. N. W. Lekkerkerker and A. Stroobants on page 822 of this issue¹, challenge this idea. They observe a smectic phase with distinguished layers and without long-range positional order of rods within the layers, without there being any attractive interaction between the rods. The only interaction in this system is one in which the cylinders cannot interpenetrate each other — the 'steric' or 'excluded-volume' interaction.

Liquid crystals consist of anisotropic organic molecules, which have one or more mesophases (intermediate phases) between the crystalline and the isotropic-liquid states. Transitions to these mesophases can be brought about by purely thermal processes (thermotropic) or by varying the concentration of solvents (lyotropic)². The most extensive characterization of the physical properties of various smectic phases has been carried out in thermotropic liquid crystals which were originally classified by G. Friedel in 1922 into three categories³: nematic, cholesteric and smectic.

In the nematic phase, the molecules are

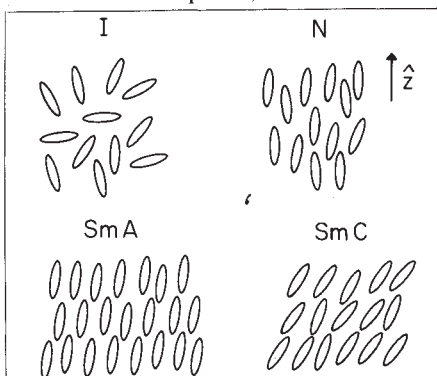


Fig. 1 Schematic molecular arrangement for isotropic (I), nematic (N), smectic-A (SmA) and smectic-C (SmC) phases in liquid crystals; $\hat{z}$, average of molecular directors.

spontaneously oriented with their long axes (molecular directors) pointing to some specific direction which distinguishes itself from the isotropic phase (Fig. 1). The nematic material has a viscosity about the same as that of light machine oil at room temperature and the molecules fill a container with no positional ordering, as in a liquid. The cholesteric mesophase is similar to the nematic phase except that it is composed of optically active molecules. As a consequence a helical structure shows up with the molecular director precessing around the normal of the molecular director. The typical size of the pitch is about 1 μm which is much larger than the width of the molecules (about 7 $\text{\AA}$).

During the past 10 years, extensive high-resolution X-ray investigations of various smectic liquid crystals have revealed the molecular arrangements within each layer. Two different categories have been identified⁴: one with long-range positional order within the layers and one without. In fact, 20 different mesophases have now been identified within the old smectic phase, and 'smectic' is now reserved for those without long-range positional order. The phase discussed¹ by Frenkel *et al.* is the smectic-A phase in the field of thermotropic liquid-crystals. On average, the molecular directors are normal to the layer and there is no long-range positional order within the layers (Fig. 1).

L. Onsager did early model calculations on the isotropic–nematic phase transition, caused by increased density, by considering a fluid of long thin rods with no force between them except the steric interaction⁵. The first attempts to address the formation of the smectic-A phase^{6,7} used an attractive pair potential between elongated molecules, as have all subsequent models.

In a previous computer study⁸ using hard spherocylinders without attraction with length-to-width ratios of 0.25 (disk-like) to 5 (rod-like), Frenkel and co-workers found that as the density increases, the molecules first align from the isotropic phase to form the smectic phase, and then crystallize onto a lattice. But between the nematic and crystalline phases of all except the most disk-like molecules, periodic oscillations arise along the mole-

cular director, strongly indicative of the formation of the smectic-A phase (Fig. 2). Although it is understandable that the repulsive steric interactions should cause the spherocylinders to align, it is not clear how they are drawn into the layered structure of the smectic-A phase without any attractive interaction.

In this issue¹, Frenkel *et al.* confirm the smectic-A phase of rod-like molecules and show that it is thermodynamically stable with respect to the isotropic, nematic phases and crystalline phases. The pre-translational smectic-layer fluctuations

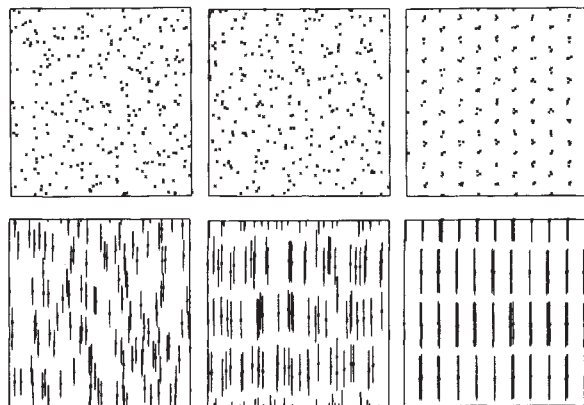


Fig. 2 The nematic (left), smectic (middle) and solid (right) phases of a system of rod-like spherocylinders without attraction. The views along the molecular directors (top) show that long-range positional ordering within the planes occurs only in the solid phase. The views from the side (bottom) show the molecules being drawn into planes as the density increases from left to right. (From ref. 8.)

seen near the nematic–smectic-A transition suggests that this transition could be continuous or weakly first-order. The present study, however, uses too small a sample to distinguish these alternatives.

One obvious extension of the present study is the smectic-C phase in which the molecular directors tilt away from the layer normal (Fig. 1). The molecular tilt angle can continuously decrease to zero as the temperature increases toward the smectic-C–smectic-A transition. This phase transition can be well characterized by a mean-field theory⁹. It remains to be seen, however, whether the steric interaction is sufficient to account for the formation of the smectic-C or whether dipolar interactions are essential. In this respect a computer simulation on a realistic object, similar to a liquid crystal molecule, should be extremely useful. □

1. Frenkel, D., Lekkerkerker, H. N. W. & Stroobants, A. *Nature* **332**, 822–825 (1988).
2. Chandrasekhar, S. *Liquid Crystals* (Cambridge University Press, 1977).
3. Friedel, G. *Ann. Phys.* **18**, 273–474 (1922).
4. Gray, G. W. & Goodby, J. W. *Smectic Liquid Crystals* (Leonard Hill, Glasgow and London, 1984).
5. Onsager, L. *Ann. N. Y. Acad. Sci.* **51**, 627–647 (1949).
6. McMillan, W. L. *Phys. Rev. A* **4**, 1238–1246 (1971).
7. Kobayashi, K. *Mol. Cryst.* **13**, 137–148 (1971).
8. Stroobants, A., Lekkerkerker, H. N. W. & Frenkel, D. *Phys. Rev. Lett.* **57**, 1452–1455 (1988).
9. Huang, C. C. & Lien, S. C. *Phys. Rev. A* **31**, 2621 (1985).

C. C. Huang is in the School of Physics and Astronomy, University of Minnesota, Minneapolis, Minnesota 55455, USA.

Chemical toxicology

How to identify a carcinogen

Alastair Hay

CHEMICALS that are likely to cause cancer by attacking DNA can be identified with some precision. Both their chemical structure and their ability to cause mutations in the bacteria *Salmonella* are almost sufficient to flag so-called genotoxic carcinogens, according to John Ashby and Ray Tennant in a paper in the latest issue of *Mutation Research*¹. Their conclusion, resulting from their survey of around 222 carcinogens and non-carcinogens, will cause relief to researchers, regulatory agencies and chemical manufacturers, as recent reports have indicated^{2,3} that the *Salmonella* assay (the so-called Ames test) does not identify all carcinogens.

Last year, Zeiger *et al.*³ surveyed 224 chemicals and reported that the Ames test has a sensitivity (percentage of carcinogens identified as mutagens) of only 54 % and a specificity (percentage of non-carcinogens identified as non-mutagens) of only 70 %. Even worse, Tennant *et al.*⁴ surveyed 73 carcinogens and non-carcinogens, and found that the *Salmonella* assay would only identify about 45 % of carcinogens.

These results are a far cry from observations made a decade ago when sensitivities and specificities of 90 % or more were claimed for the Ames test⁵⁻⁷. People then believed that the Ames test would correctly identify carcinogens and non-carcinogens, making the expensive two-year animal carcinogenicity tests unnecessary. The Ames test is cheap and simple: all that is required is to plate an unknown chemical onto agar containing *Salmonella* and to look for mutation, which is demonstrated by growing colonies of the bacteria. Positive results label the chemical a probable carcinogen, providing an answer in days rather than years.

Even ten years ago, it was noted that the Ames test missed some potent carcinogens⁸. What was needed was another *in vitro* short-term test, perhaps even a battery of tests, to pick out those chemicals missed. By creating a net of even finer mesh, it was argued, it would be possible to screen all chemicals in the knowledge that none, or only very few, would escape.

Sadly, the evidence suggests that the net does work for certain chemicals, particularly those that become reactive electro-

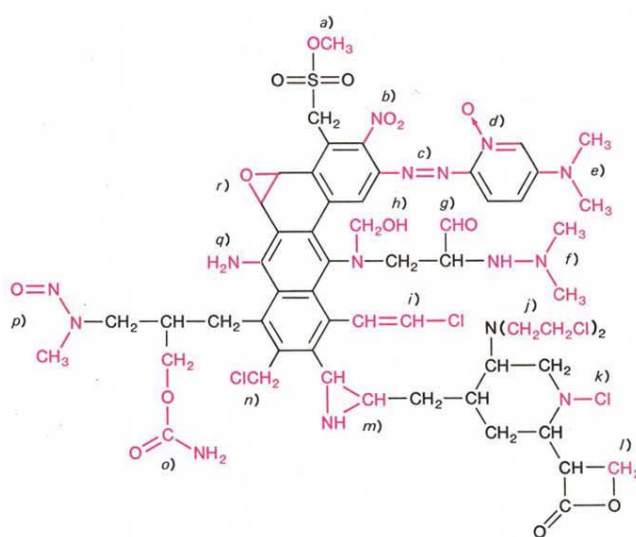
philes⁸ and attach to DNA, but it certainly does not work for all of them¹. The electrophilic portion of the molecule is an electron-deficient region present either because of the particular structure of a chemical, or as a result of metabolic activation. This electrophilic region accepts electrons from nucleic acids in DNA, allowing the chemical to react and combine with it, leading to mutation.

In their criticism of the sensitivity of the Ames test, Tennant *et al.*⁴ also evaluated

results for 115 carcinogens, 24 equivocal carcinogens and 83 non-carcinogens. *Salmonella*, they argue, is a sensitive method for picking out chemicals that are genotoxic and could attack DNA. Because of the agreement between analysis of chemical structure and *Salmonella* they conclude that, in combination, the two methods reveal two groups of carcinogens, those that are genotoxic and those that cause cancer in other ways. For the low genotoxic carcinogens that are negative in the Ames test (for example, benzene) the authors recommend a second genotoxicity assay.

To get around the problem of different test protocols, Ashby and Tennant used data from Ames tests conducted in one laboratory using a standard protocol.

They compared this information with the results of rodent carcinogenicity data on 222 chemicals evaluated by the National Cancer Institute/National Toxicology Program. In explaining the apparent decreasing sensitivity of the Ames test over the past decade, Ashby and Tennant conclude that this was largely caused by the nature of the chemicals under study. When the Ames test was first validated in the 1970s, most of the chemicals tested were both potent carcinogens and mutagenic. Subsequently, more non-genotoxic carcinogens have been added to the National Toxicology Program list as a result of animal studies, and it was perhaps inevitable that these would be missed by the Ames test because they do not form reactive electro-



A hypothetical chemical showing main structural units (red) which would lead to a chemical being classed as a potential carcinogen. The units in red are regions which would react with DNA, based on identical or comparable carcinogen structures in animals. From ref. 1.

three of the better-known short-term tests: those for chromosome aberrations; for sister chromatid exchange; and a mutagenicity assay using mouse lymphoma cells. These authors found that, although each was more sensitive than the Ames test, they were all less specific, and therefore falsely labelled more non-carcinogens as probable carcinogens. Equally damaging was their observation that no combination of the four tests is any better than *Salmonella* on its own for flagging probable carcinogens. This looked disastrous for carcinogenicity screening. It seemed increasingly likely that expensive animal carcinogenicity studies were the only way to identify carcinogens.

The new work¹ of Ashby and Tennant (the same author who forced re-evaluation of the Ames test) lifts this gloom. From their comparison, Ashby and Tennant conclude that there is about a 90 % correlation between structural analysis — looking at reactive sites on a chemical (see figure) — and *Salmonella* assay

results. Non-genotoxic carcinogens are thought to operate by a range of other mechanisms such as hormonal changes, induced in the thyroid gland by several thyroid-specific carcinogens. Such carcinogens may present a much reduced hazard to man, or even no hazard at all.

About a third of the 83 non-carcinogens investigated by Ashby and Tennant are mutagenic to *Salmonella*. This finding suggests that a significant proportion of *in vitro* genotoxins are not carcinogenic, perhaps because they are not absorbed, or because they are detoxified *in vivo*. To classify these chemicals correctly — initially highlighted by structural analysis and *Salmonella* testing as probable carcinogens — Ashby and Tennant suggest using a second genotoxicity assay, conducted *in vivo*. One such test is the mouse micronucleus assay, which relies on cells with chemically induced chromosome aberrations having an unusual distribution of chromatin during cell division, which can be observed as distinct micronuclei in

the cytoplasm. Validation of this genotoxicity assay, according to Ashby and Tennant, is likely to be of more use than the *in vitro* tests which do not aid identification of carcinogens.

Life-time carcinogenicity studies will still be needed, however, for those non-genotoxic carcinogens that are tissue, sex or species specific, in contrast to the genotoxic carcinogens that cause tumours in different species and at multiple sites, which will need only a limited bioassay programme. This second group of reactive compounds will be identified through their structure, their effect in *Salmonella*, and a second *in vivo* or *in vitro* genotoxicity assay, and it will probably be sufficient to conduct animal tests using male rats and female mice rather than both sexes of both species in order to confirm them as animal carcinogens.

The new work by Ashby and Tennant makes the National Toxicology Program database widely available for the first time. Perusal of the information therein raises many questions, only a few of which can be dealt with here. One concerns the variability in the percentage of control animals with tumours. The data on three chemicals, Dicofof, 3-nitro-*p*-acetophenide and piperonyl sulphoxide, show 17, 22 and 33%, respectively, of male control mice with liver tumours. This is almost a dose-response effect in itself, and it would be interesting to know why this happens.

Second, where there are only haematopoietic tumours in a single sex or species, and where the tumour incidence could only just be described as a dose-response effect — such as with the non-genotoxins butyl benzyl phthalate (a plasticizer) and the dye CI vat yellow⁴ — the question arises whether the same tumour pattern would be observed if the studies were repeated. Are the tumour data real, or are they a one-off observation?

Some halogenated chemicals are carcinogenic in animals, but others are not. Ashby and Tennant investigate how a combination of structural analysis and *Salmonella* data can identify those that are genotoxic carcinogens yet fail to identify the others which cause cancer by some other mechanism. Simple mono-carbon halogens present particular problems that cannot be explained by structural analysis. For example, neither chloroform (CHCl_3) nor dibromochloromethane (CHBr_2Cl) are mutagenic in the Ames test, yet both are carcinogenic, whereas iodoform (CHI_3), which also has three halogen substituents, is mutagenic but not car-

cinogenic. Dichloromethane (CH_2Cl_2) has not been tested in the Ames test but is undoubtedly a carcinogen. Structural analysis of these four chemicals does not identify them as potential carcinogens, suggesting that the carbon atom is heavily shielded by the bulky halogen substituents and hence unavailable for combination with DNA. Perhaps metabolism of iodoform *in vivo* accounts for its non-carcinogenicity, and it could be that the other three chemicals are examples of non-genotoxic carcinogens.

For the moment it seems that there are no helpful rules for these simple halogen alkanes other than that animal tests are

essential to give a clear indication of their carcinogenicity. And what is true for these halogens is true for many other chemicals. Animal tests, unfortunately, still remain the one sure way of obtaining information about carcinogenicity. But the right short-term tests can cut the number of animals used and identify the most hazardous genotoxic carcinogens. Supporting this, Shelby⁹, in the same issue of *Mutation Research*, reports that 21 of the 23 established human carcinogens are detected by the above approach. □

Alastair Hay is in the Department of Chemical Pathology, University of Leeds, Leeds LS2 9JT, UK.

Solid-state physics

Polyexcitons in ultrapure silicon

Peter Y. Yu and C. Kittel

A NEW kind of electronic excitation, the polyexciton, has been discovered in ultrapure silicon by Steele *et al.*¹ A polyexciton comprises several electron-hole pairs. This observation is noteworthy, because silicon is such an important material and because excitons are perhaps the most fundamental excitation in all non-metallic solids, from semiconductors to chlorophyll. The potential impact of this observation has yet to be assessed.

An exciton is a neutral excited state of a solid in which an electron (e^-) is raised in energy to an unoccupied state, leaving behind a hole (h^+) in a band of energy otherwise filled with other electrons. The electron and hole can be bound together as e^-h^+ by their Coulomb attraction, forming an entity that is the analogue of a hydrogen atom or a positronium atom (made of an electron and a positron). The exciton, if mobile, differs from other analogues of hydrogen atoms in solids, such as impurity atoms that donate or accept electrons. But the similarity between all these objects suggested to Lampert² that there could exist stable analogues of H_2 as excitonic molecules $\text{X}_2 = e^-h^+$, or as excitons bound to a donor (D^+e^-) as $e^-h^+\text{D}^+e^-$ or to an acceptor.

In some semiconductors (technically, those with multivalley or degenerate band edges) the Pauli exclusion principle (that no two electrons can be in the same state) is relaxed to enhance the stability of X_3 , X_4 , ... — the analogues of the trimers, tetramers and so on of the hydrogen atom³. The stability can be explained by considering n equivalent band edges (the limits of permissible energy): an 'orbital' of hydrogen in this environment is only filled in the Pauli sense when it is occupied by $2n$ electrons, rather than by the two electrons that fill the orbital in free space. In silicon $n=6$ for the conduction band and $n=2$ for the valence band (Fig. 1). Morgan

considered⁴ the polyexciton problem in more detail and concluded that "it appears probable that stable complexes up to at least X_6 in silicon can be formed". Steele *et al.* present¹ experimental evidence for polyexcitons up to X_5 .

There are two relevant features of the band structure (the ranges of allowed electron energy and momentum) of silicon. First, the band gap is indirect because the minimum possible energy in the conduction band occurs at a different value of the 'crystal' momentum from the maximum of the valence band. By contrast, in a direct-gap semiconductor such as GaAs, the extrema of the conduction and valence bands occur at the same value of the crystal momentum, actually at zero just as for a particle in free space. Second, in silicon there can be 6×2 electrons and 2×2 in the lowest orbitals; in GaAs there can be 1×2 electrons and 2×2 holes. The second factor of 2 arises, as usual, from two states of electron spin.

Hybrid or bound polyexciton complexes were reported in silicon more than 10 years

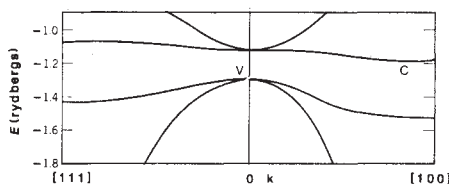


Fig. 1 Energy-band structure of silicon. The energy E is plotted on the vertical axis and the electron crystal momentum k (along the crystallographic [111] and [100] directions) on the horizontal axis. The lowest energy in the conduction band is labelled C, and by crystal symmetry there are six minima equivalent to C. The highest energy in the valence band is labelled V and two bands coincide at this point. C and V occur at different values of k , so silicon is termed an indirect-gap semiconductor. For a direct-gap semiconductor, such as GaAs, minimum C occurs vertically above V at $k=0$.

1. Ashby, J. & Tennant, R.W. *Mut. Res.* **204**, 17–115 (1988).
2. Dunkel, V.C. *et al. Environ. Mutagen.* **7**, Suppl. 5 (1985).
3. Zeiger, E. *Cancer Res.* **47**, 1287–1296 (1987).
4. Tennant, R.W. *et al. Science* **236**, 933–944 (1987).
5. McCann, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 5135 (1975).
6. McCann, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 950 (1975).
7. Purchase, I.F.H. *et al. Nature* **264**, 624–629 (1976).
8. Miller, J.A. & Miller, E.C. in *Origins of Human Cancer* (eds Hiatt, H.H. *et al.* 605 (Cold Spring Harbor, New York, 1977).
9. Shelby, M.D. *Mut. Res.* **204**, 3–15 (1988).

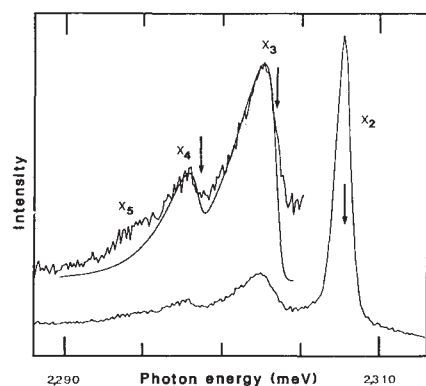


Fig. 2 Photoluminescence spectrum of silicon at 20 K. The solid curve is a fit to the triexciton and tetraexciton lines, with amplitude expanded four times. The three vertical arrows mark the transition energies of X_2 , X_3 , and X_4 , all at zero kinetic energy. The peak intensity measured in this spectral region was 4 counts per channel per minute. (After ref. 1.)

ago⁵. It took much longer to observe free polyexcitons because of two difficulties. Ultrapure silicon is required so that excitons will be trapped by each other rather than by impurities. And the conclusive evidence of these polyexcitons is photon emission from the simultaneous recombination (the electrons 'falling' back into the valence band) of two of the excitons⁶. Such emission is extremely weak, having a rate orders of magnitude lower than that of single exciton recombination. Further, the photon emitted by two-exciton recombination is strongly reabsorbed by the semiconductor because its energy is higher than the bandgap. Steele *et al.*, working with the most sensitive radiation detectors available, took 20 hours to obtain one emission spectrum from two-exciton recombination (Fig. 2).

Polyexcitons are stable in silicon in a narrow temperature range only — 20–50 K. At higher temperatures they decompose thermally; at lower temperatures they condense into an electron–hole liquid. Because of this narrow stability range it is unlikely that they will have any immediate application in silicon technology. But they will have an impact in the physics of small clusters, in semiconductor physics in general, and they will stimulate more detailed theoretical work than in the past when their existence was speculative. Like small clusters of atoms, polyexcitons are a bridge between simple excitations, of single excitons and the many-particle excitations of the electron–hole liquid. □

1. Steele, A.G., McMullan, W.G. & Thewalt, M.L.W. *Phys. Rev. Lett.* **59**, 2899–2902 (1987).

2. Lampert, M.A. *Phys. Rev. Lett.* **1**, 450–453 (1958).

3. Wang, J.S. & Kittel, C. *Phys. Lett.* **42A**, 189–190 (1972).

4. Morgan, T.N. *Nuovo Cim.* **39B**, 602–608 (1977).

5. Thewalt, M.L.W. in *Excitons* (eds Rashba, E. J. & Sturge, M. D.) 394 (North-Holland, Amsterdam, 1982).

6. Schmid, W. *Phys. Rev. Lett.* **45**, 1726 (1980).

Peter Y. Yu is Professor of Physics and C. Kittel is Professor Emeritus at the University of California at Berkeley, California 94720, USA.

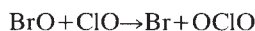
Atmospheric chemistry

Causes of ozone depletion

Brian Thrush

IN the past decade, much effort has been put into laboratory studies of the chemical reactions that control stratospheric ozone. A constant question was whether all the important processes and chemical species had been incorporated in the models. Nevertheless, the Antarctic ozone hole was totally unpredicted. Chlorine compounds were strongly implicated but the accepted cycle $\text{Cl} + \text{O}_3 \rightarrow \text{ClO} + \text{O}_2$; $\text{ClO} + \text{O} \rightarrow \text{Cl} + \text{O}_2$ could not account for the rapid ozone loss in the lower stratosphere. Several sequences of chemical reactions that regenerate chlorine atoms have now been invoked to explain the rapid decay during spring of ozone trapped in the Antarctic polar vortex¹. The most generally favoured is that suggested by Molina and Molina². This involves the dimerization of ClO radicals at very low temperature to form Cl_2O_2 , which is then broken down by ultraviolet light, regenerating chlorine atoms which destroy ozone. On page 796 of this issue³, R.A. Cox and G.D. Hayman identify Cl_2O_2 in laboratory experiments. Their work supports this mechanism, but it also raises further problems about the complex and confusing chemistry of the unstable chlorine oxides.

Elucidating the chemistry of these systems in the atmosphere and in the laboratory involves the common difficulty that one is largely dependent on visible and ultraviolet spectroscopy for identifying trace species. Fortunately, ClO and OClO have very strong, characteristic, banded spectra, but other chlorine oxides such as ClOO and Cl_2O_2 show broad continua. The atmospheric measurements are clear: they show abnormally high amounts of ClO in the lower stratosphere where ozone has decayed rapidly in the Antarctic ozone hole^{4,5}. Here about one-third of the total chlorine is present as ClO, which approaches 1 part per billion (10^9), about 100 times that predicted by conventional atmospheric models. OClO is also detected⁶, but in much smaller amounts which are consistent with its formation from the reaction



This reaction is one of the few gas-phase processes which are known to yield OClO, and it also releases bromine atoms which destroy ozone efficiently. But the very small amounts of bromine compounds in the stratosphere cannot account for the rapid decay of ozone there.

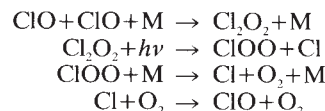
Much more interest has centred on the thermodynamically stable isomer ClOO, which is also an important atmospheric

species. The first clear evidence for the existence of ClOO came from the work of Porter and Wright⁷ who used an intense flash of light to generate Cl atoms from Cl_2 in the presence of molecular oxygen. These authors showed that the ClO which they detected spectroscopically must have been formed via the intermediate ClOO. This result was confirmed by Johnston⁸ who, like Cox and Hayman³, used repeated pulses of light to produce modulated spectra of transient species. In this way Johnston obtained infrared and ultraviolet spectra of ClOO in the $\text{Cl}_2 + \text{O}_2$ system.

Surprisingly, the latter spectrum is identical with the ultraviolet spectrum of Cl_2O_2 reported by Cox and Hayman in this issue³ apart from the extinction coefficient, which is a factor of 2 greater, a difference that could have arisen from the uncertainty of the amount of the transient species of Johnston's work. Did Johnston observe ClOO or Cl_2O_2 ? His infrared spectra favour the former interpretation. On the other hand, the infrared spectra obtained by Molina and Molina², accompanying an electronic spectrum of Cl_2O_2 with a maximum at a wavelength of 270 nm rather than 240 nm, correspond to a Cl_2O_2 species containing at least four atoms.

The intriguing question then is why do the ultraviolet spectra of ' Cl_2O_2 ' obtained by Cox and Hayman³ and by others⁹ vary so much depending on source of ClO used? The idea that there are two isomers, ClOOCl and ClOClO, now seems less plausible than the transitory formation of Cl_2O_2 absorbing at these wavelengths in systems where ClO is present together with OClO, an interpretation which is scarcely new¹⁰.

The techniques are available for these questions to be sorted out by a combination of infrared and ultraviolet spectroscopy. In the meantime, it looks as though Cox and Hayman have successfully measured the properties of ClOOCl and that the mechanism of the rapid ozone decay in the Antarctic spring is



($h\nu$ is a photon of light and M an unreactive molecule), although the long-wavelength tail of the Cl_2O_2 spectrum has not yet been determined well enough to deduce whether or not this mechanism would be sufficiently fast with the low spring Sun.

Apart from the difficulties of carrying out laboratory studies of gas-phase reactions at temperatures as low as the 180 K encountered in the lower stratosphere in Antarctica, laboratory studies are also needed of the reactions which must occur on the aerosol particles of the polar stratospheric clouds which are present at times of rapid ozone decay. These ice particles are probably nucleated by nitric acid trihydrate, and it is believed that reactions on their surface help to keep chlorine in a readily activated form rather than as the relatively inert HCl. Is the heterogeneous reaction $\text{ClONO}_2 + \text{HCl} \rightarrow \text{HNO}_3 + \text{Cl}_2$ one such important process? $\square$

1. Pyle, J.A. & Farman, J.C. *Nature* **329**, 103–104 (1987).
2. Molina, L.T. & Molina, M.J. *J. phys. Chem.* **91**, 433–436 (1987).
3. Cox, R.A. & Hayman, G.D. *Nature* **332**, 796–800 (1988).
4. de Zafra, R.L. *et al.* *Nature* **328**, 408–411 (1987).
5. Solomon, P.M. *et al.* *Nature* **328**, 411–413 (1987).
6. Solomon, S. *et al.* *J. geophys. Res.* **82**, 8329–8338 (1987).
7. Porter, G. & Wright, F.J. *Discuss. Faraday Soc.* **14**, 23–24 (1953).
8. Johnston, J.S., Morris, E.D. & Van den Bogaerde, J. *J. Am. chem. Soc.* **91**, 7712–7727 (1969).
9. Basco, N. & Hunt, J.E. *Int. J. chem. Kinetics* **XI**, 649–664 (1979).
10. Lipscomb, F.J., Norrish, R.G.W. & Thrush, B.A. *Proc. R. Soc. A233*, 455–464 (1956).

Brian Thrush is in the Department of Physical Chemistry, Cambridge University, Lensfield Road, Cambridge CB2 1EP, UK.

Pattern formation

In my beginning is my end

Geoffrey North

THE ends of insect embryos play a fundamental role in organizing their development from the earliest stages, and in the case of the fruitfly *Drosophila melanogaster* many of the components of this process have in recent years been identified genetically. The results reported at a recent meeting* begin to show, from a combination of studies of genetic interactions and the analysis of the products of the genes and their spatial distribution, how three systems interact to map out the early embryo. The protein product of *bicoid*, the key gene in the determination of anterior structures, is expressed as a morphogenic gradient throughout the egg and early embryo. And while the system responsible for organizing development with respect to the posterior end of the fruitfly is less well understood, it is intriguing that a gene thought to respond to a signal from an organizing centre at this end turns out to encode what looks very much like a steroid hormone receptor.

To make the significance of the new results clear a little background knowledge is required. Classical manipulative experiments with insect eggs and embryos led Klaus Sander to the view¹ that by the time the egg is fertilized two organizing centres have already been established, one at either end of the egg, that determine the polarity of the embryo along the anteroposterior axis by providing spatial cues interpreted by the progeny of the zygotic nucleus.

Christianne Nusslein-Volhard's group at the Max-Planck Institute for Developmental Biology in Tübingen has shown² that Sander's conclusions apply to the fruitfly, allowing a genetic analysis of the components of the polarity-organizing centres that has proved remarkably

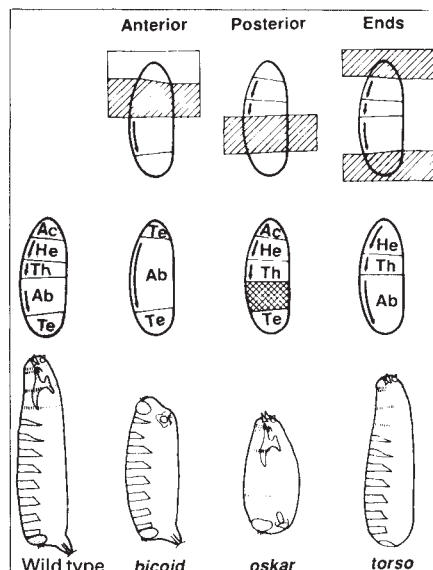
successful³. The study of maternal-effect mutations that disrupt the development of the fruitfly has in fact revealed three distinct systems (see figure) operating to organize pattern along the anteroposterior axis of the embryo.

Anterior system

One of these is the anterior-organizing centre revealed by mutations in the *bicoid* gene. The embryonic progeny of female fruitflies homozygous for *bicoid* mutations lack head and thorax, just like the embryos that develop from eggs from which the anterior cytoplasm has been allowed to escape. Such mutants can be rescued by transplanting anterior cytoplasm from wild-type embryos. An important observation from such transplantation experiments is that the head structures form at the site of transplantation, providing an initial clue that the *bicoid* gene product has a direct morphogenic role.

The *bicoid* gene has been cloned^{4,5}, allowing the distribution of its RNA and protein products to be determined in eggs and embryos. Like other maternal-effect genes, *bicoid* is expressed in the nurse cells clustered at the anterior end of the developing oocyte. The *bicoid* RNA is transported from the nurse cells into the oocyte at its anterior end, and *in situ* hybridizations have shown it stays tightly associated with that end of the embryo for as long as it can be detected. Two genes, *exuperantia* and *swallow*, have been implicated in localizing *bicoid* activity at the anterior end of embryos, and in mutants for these genes *bicoid* RNA can be seen to have spread⁵ from the anterior end to form a nearly homogeneous distribution.

Nusslein-Volhard and co-workers have now obtained antibodies against the protein product of the *bicoid* gene which they have been able to use to see where it is expressed in embryos in relation to the



The three phenotypic classes of *Drosophila* embryo. Top row, the normal origin of regions deleted in mutant embryos, indicated by hatching. In *bicoid*, the acron is transformed into a telson (open rectangle). Middle row, the corresponding changes in the fate maps of the remaining areas of the embryos. Ac, acron; He, head; Th, thorax; Ab, abdomen; Te, telson. Bottom, schematic drawings of phenotypes. From ref. 3.

bicoid RNA. It turns out that the *bicoid* protein forms a roughly exponential gradient from the anterior to the posterior end of fruitfly embryos. By looking at how this gradient is affected in various mutants in which pattern formation along the anteroposterior axis is disrupted, a clear correlation is found between the *bicoid* protein concentration at a particular position and the structures formed by the progeny of the cells occupying the position.

This correlation, Nusslein-Volhard argues, provides strong support for the view that *bicoid* protein is forming a morphogenic gradient in the classical sense, with the variation in concentration of the protein providing direct spatial information telling cells where they are along the anteroposterior axis of the embryo. Some clues as to how this gradient may be interpreted come from the sequence of the *bicoid* protein and a study of zygotically expressed segmentation genes.

The *bicoid* gene has the 'homoeobox' sequence typical of the homoeotic genes that specify the segments of the fruitfly and which is known to confer the ability to bind DNA on proteins that contain it. The clear implication is that the *bicoid* protein product regulates the expression of genes in the descendants of the zygotic nucleus, which it should be remembered take up their positions at the periphery of a fruitfly embryo in what is a syncytium before the nuclei become enclosed when cellularization takes place.

A good candidate for regulation by *bicoid* is the gap gene *hunchback*. Gap genes are a class of zygotic genes thought

*The joint annual meeting of the British Societies of Cell and Developmental Biology was held at the University of Bristol, 12–14 April 1988.

to be first in a hierarchy responsible for segmentation and segment specification. Gap mutants lack contiguous groups of segments, and *hunchback* mutants in particular lack anterior segments including head structures. The *hunchback* gene is expressed as a band at the anterior end of fruitfly embryos that is absent in *bicoid* mutants⁶.

Posterior system

The two other systems responsible for organizing pattern along the anteroposterior axis of fruitfly embryos have both been identified by small sets of genes that have very similar mutant phenotypes. One is the posterior-organizing centre, dependent on a set of genes of which the prototype is termed *oskar*, and the other is a system responsible for the formation of terminal structures dependent on the *torso* class of genes.

Cytoplasmic removal and transplantation experiments show that, like the anterior organizing centre, the posterior centre has a global role in the organization of embryonic pattern. There are significant differences between the two systems, however. One is that although the activity for the rescue of mutants of the *oskar* class is localized at the posterior embryonic pole, to rescue the mutants wild-type posterior cytoplasm must be transplanted into the prospective abdominal region, not the posterior pole itself. A second difference is the relative lack of influence of the site of transplantation of posterior cytoplasm on the position where posterior structures develop.

Genetic and transplantation experiments led to the view that the *oskar* group of genes cooperate to generate a signal at the posterior pole that is transmitted to the abdominal region where it acts, possibly by activating the expression of the gap gene *knirps*. The cooperation was inferred from the observation that posterior cytoplasm from mutants for one gene of the *oskar* group cannot rescue mutants for another gene of the same group (with one exception). Ruth Lehmann (Laboratory of Molecular Biology, Cambridge), however, has now discovered in experiments carried out in collaboration with Sander that rescuing activity is present in the nurse cells of all but one of these mutants. Mutants for the gene *nanos* are the exception, implicating the product of the *nanos* gene as the posterior signal itself. The other genes are thought to be involved in transporting the *nanos* signal to the posterior pole from the nurse cells, and later in transmitting a signal from the posterior pole to the abdomen.

The lack of influence of the site of transplantation of posterior cytoplasm on where the posterior structures develop, mentioned above, is revealed when double-mutants for the posterior and anterior organizing centres are transplanted

with the wild-type posterior cytoplasm: in this case, 'double-abdomen' embryos are formed with posterior poles at either end, just like *bicaudal* mutants, even if the posterior cytoplasm is transplanted into the middle of the mutant embryos. If, however, the transplanted embryo lacks not only the posterior and anterior but also the terminal organizing system, the result is different.

Lehmann reported that when posterior cytoplasm is transplanted into the middle of triple mutants for *bicoid*, *oskar* and *torso* then a posterior pole is formed at the site of transplantation, and a mirror-image embryo is formed that is the exact reverse of when *torso* activity is present. The implication is that there is some kind of interaction between the terminal and posterior systems. Such an interaction was in fact hinted at by some earlier work of Sander which led him to postulate that a kind of 'polar focusing' takes place to orient the posterior organizing centre with respect to the posterior terminus of the embryo. Whether this occurs directly at the level of the maternal gene products, or at a later stage involving interactions between the zygotic gap genes, is at present unclear.

As none of the genes of the *oskar* group has yet been sequenced, there are at present few clues as to how they work. The similarity of the phenotype of *oskar* mutants to that of mutants for the gap gene *knirps*, however, suggested that the signal from the posterior-organizing centre is needed to activate *knirps* expression in the prospective abdominal region of the embryo. Herbert Jackle (Max-Planck Institute for Developmental Biology, Tübingen) reported that the *knirps* gene has at last been cloned by his group, and the sequence of the gene reveals that its protein product has a domain that clearly puts it in the class of DNA-binding proteins characterized by the mammalian steroid-hormone receptors. This is a particularly exciting result in the wake of the recent finding that the receptor for retinoic acid, implicated in the specification of the anterior-posterior digit pattern of the chick wing, is a member of this class of proteins^{7,8}. Whether the signal from the posterior organizing centre of fruitfly embryos is a ligand that activates the *knirps* protein, rather than an activator of *knirps* gene expression as hitherto thought, is not known, but an answer to this question should not be long in coming. □

1. Sander, K. *Adv. Insect. Physiol.* **12**, 125 (1976).
2. Fröhnhoffer, H. G. & Nusslein-Volhard, C. *Nature* **324**, 120 (1986).
3. Nusslein-Volhard, C., Fröhnhoffer, H. G. & Lehmann, R. *Science* **238**, 1675 (1987).
4. Frigerio, G. *et al.* *Cell* **47**, 735 (1986).
5. Berleth, T., Noll, M. & Nusslein-Volhard, C. *EMBO J.* (in press).
6. Tautz, D. *Nature* **332**, 281 (1988).
7. Petkovich, M. *et al.* *Nature* **330**, 444 (1987).
8. Giguere, V. *et al.* *Nature* **330**, 624 (1987).

Geoffrey North is Deputy Biology Editor of Nature.

Daedalus

Cylinders of garbage

EVERYTHING burns completely in high-pressure oxygen. This is the principle of the oxygen-bomb calorimeter, which has burnt its way through the vast array of chemical substances in the thermochemical tables, and the huge variety of soggy and aqueous foodstuffs in dieters' calorie charts. Now Daedalus has a new use for the principle — waste disposal.

Almost all urban waste is combustible: paper, plastics, food residues, household grime, even aluminium and sheet-steel. Some urban authorities burn it already, under big, expensive, steam-raising boilers. But Daedalus will use the diesel principle. The cylinder of an oxygen-breathing diesel engine, reaching many tens of atmospheres and many hundreds of degrees Celsius on its compression stroke, would be an irresistibly oxidizing environment.

The diesel waste-combustor will need a special fuelling system. Refuse can hardly be ground up and injected into its cylinders as a fine spray. Daedalus's engine is inverted, with the cylinders at the bottom. A charge of rubbish is just pushed into a cylinder, where it rests on the cylinder-head floor; the entry port is sealed. At each compression stroke the engine takes a 'bite' at this fuel, burning as much as it can with one cylinderful of oxygen. So it runs continuously, breathing in oxygen and expelling burnt gas, until the whole charge of trash has been consumed. The entry port then opens and rams a fresh charge into the cylinder, while expelling any ash from the previous charge. Recharging each cylinder may take several revolutions, during which time the engine will be driven by the remaining cylinders. The brief loss of power should not be serious.

The diesel waste-combustor will be worn away rapidly by its abrasive fuel, and may also tend to burn its own structure. Fortunately, the advanced ceramic materials now being developed for military diesel engines may permit a design which is free of such troubles. The ash from the engine will be mainly glass, ceramics and metal oxides, probably as fine dust and clinker. It may turn out to be convenient to copy iron-smelting practice and add a limestone 'flux' to the fuel, to melt the ash to a liquid slag. It could even be blown by the engine-exhaust to that useful insulator, slag-wool.

Such refinement makes most sense for big municipal installations, burning a whole city's rubbish. But the diesel waste-combustor may make the whole idea of urban rubbish collection obsolete. A small household unit could enable each family to burn its own trash, and use the resulting power and waste heat itself. Daedalus has great hopes of his small 'diesel dustbin'.

David Jones

Groups and grandmothers in neuroscience

SIR—In his review of *Neural Darwinism: The Theory of Neuronal Group Selection* (*Nature* 331, 571; 1988) Horace Barlow has made several factual errors and has misrepresented the theory by misinterpreting the nature of neuronal groups. Barlow's chief objection is that neuronal groups cannot carry out the functions proposed by the theory. This misinterpretation arises because he does not understand that the major mechanism of selection acts upon synapses in populations¹. Barlow says that the theory uses Hebb's rule for synaptic plasticity. In fact, the book states explicitly (p.201) that these populations do not, in general, obey hebbian rules. This misunderstanding by Barlow invalidates all of his dependent arguments: in fact, increasing numbers of cells do not have to be incorporated into groups, experience makes the cells in a group more selective not less, and neuronal group selection confers a number of advantages that have been clearly outlined in the book. Not the least of these is that it accounts for the dynamic organization of topographic maps².

Barlow is also confused about how receptive fields are represented by cells in groups. Receptive fields are similar within a group but not identical. Moreover, the purpose of cooperativity within a group is to provide sharp functional boundaries in an otherwise diverse and overlapping anatomy. The combinatorics afforded lead to an immense diversity: admittedly, the diversity is not as immense as would obtain from permuting individual neurons, but it is overwhelmingly larger than could be represented by Barlow's concept that individual neurons each "correspond[s] to a pattern of external events of the order of complexity of the events symbolized by a word"³ — the infamous 'grandmother' neuron.

Every reviewer has an inalienable right to sins of omission. This perhaps accounts for Barlow's overlooking the main themes of the book — the central importance of perceptual categorization, the constraints of development and evolution on brain function, the role of variability in the nervous system, and the various mechanisms of re-entry between maps. One can only suspect that his actual sins of commission in the review are motivated less by the presumption that the theory "ushers in a new era in neuroscience" than by the premonition that an old one is about to be ushered out.

LEIF H. FINKEL

*The Rockefeller University,
New York, New York 10021, USA*

1. Finkel, L.H. & Edelman, G.M. *Proc. natn. Acad. Sci. U.S.A.* 82, 1291–1295 (1985).
2. Pearson, J.C., Finkel, L.H. & Edelman, G.M. *J. Neurosci.* 7, 4209–4223 (1987).
3. Barlow, H.P. *Perception* 1, 371–394 (1972).

BARLOW REPLIES—I found the book hard to understand, and the extravagant claims made for it perplexing, so I cannot dismiss the possibility that I failed to pick up some important messages. I am not convinced that this is so, but if it is I apologize and we must leave it to other, unbiased, readers to decide whether this was entirely my fault, or whether the book itself was to blame.

There are other bits of my review for which I do not apologize. Part of the book is concerned with background material which is unoriginal and adds no new insight, so I did not draw attention to it. I did mention the review of Edelman's own work on cell adhesion molecules, but it is disappointing that the work of other groups was not discussed. In the section on development there is no mention of work such as that of Willshaw, von der Malsberg or Swindale, which is certainly relevant to any discussion of the factors moulding the brain during development. It is perhaps unfair to expect an account of the state of recent computer simulations of modifiable neural networks, but an uninformed reader might gain the impression that Edelman and his colleagues are the only ones with interests in this area, and that is unfortunate.

Finkel implies that I am wedded to, or even responsible for, the notion of 'grandmother' neurons. If he would re-read the

article he quotes he would find that its ideas are less restrictive than he thinks: the cells postulated are thought to owe their properties partly to modification by experience, and room is left for cooperative or connectionist factors to mould the neurons that categorize perceptions. Room is also left for neurons that are selectively sensitive to highly specific stimuli, such as the faces of particular individuals. Finkel has a right to regard such neurons as "infamous", but I hope this does not blind him to the experimental evidence of Gross, Rolls, Perrett and their colleagues^{1–3} that they exist.

Although I do not agree with the ideas in *Neural Darwinism* I think the title is splendid, and it should encourage all of us to seek a role for selection in neurobiology. Experimental techniques are developing rapidly, and we need as many clearly expressed theories as possible about the way the brain performs its remarkable functions; the facts elicited by testing them will then weed out errors and select the fittest ideas with greater finality than any amount of discussion in these columns.

HORACE BARLOW

*The Physiological Laboratory,
University of Cambridge,
Cambridge CB2 3EG, UK*

1. Gross, C.G., Rocka-Miranda, C.E. & Bender, D.B. *J. Neurophysiol.* 35, 96–111 (1972).
2. Perrett, D.I., Rolls, E.T. & Caan, W. *Expl Brain Res.* 47, 329–342 (1982).
3. Perrett, D.I. *et al. Hum. Neurobiol.* 3, 197–208 (1984).

Convergent evolution of lysozyme sequences?

SIR—As Stewart *et al.*¹ have pointed out, there are theoretical reasons to expect the evolution of protein sequences to be divergent, and there exists abundant experimental evidence from many kinds of protein to suggest that it is. The theoretical argument applies with particular force to the bacteriolytic function of lysozyme, which can be imitated with random copolymers of glutamate and phenylalanine^{2,3}. As there are many more than twenty families of proteins for which at least six sequences are available for comparison, one ought to be surprised if one of them did not display characteristics with a likelihood less than 5%, and so to be convinced of the reality of convergence in the evolution of sequences one can hardly be satisfied with a result significant in a 95% confidence test.

The sequences of lysozyme from langur, baboon, human, rat, cattle and horse contain four loci at which the langur and cattle enzymes share identical residues not found in any other vertebrate lysozymes apart from other ruminant or colobine stomach enzymes, whereas other pairs from the six sequences share either one such identity or none¹. As the seven identities could in principle be distributed

at random among 15 pairs of sequences, one can readily calculate the probability that four or more would occur by chance in the same pair of sequences as $15p^4(p^3 + 7p^2q + 21pq^2 + 35q^3) = 0.0088$, where $p = 1/15$ and $q = 14/15$. This is smaller than implied by the reported significance in a 95% test¹, but is too large to exclude the possibility that one is dealing with more than the expected tail of the distribution.

When the lysozyme sequences were analysed on the assumption that the true phylogenetic tree linking them is the ordinary biological tree in which the langur is more closely related to the baboon than to cattle, five amino-acid substitutions were placed on the lineage leading to langur from the ancestor unique to langur and baboon. The probability of observing as many as five on this lineage was also assessed as less than 5%. But the particular placing of substitutions represents an interpretation of the observations, not the observations as such, and the putative substitutions cannot be analysed statistically as if they were actually observed. At locus 50, for example, the occurrence of glutamate in cattle and langur but glutamine in the rat and baboon was interpreted as two

parallel replacements of glutamine by glutamate in the langur and cattle lineages, though the data were also consistent with two parallel replacements of glutamate by glutamine in the rat and baboon lineages. It could, of course, be argued that it is more parsimonious to take glutamine rather than glutamate as ancestral, because then glutamine in the rat can become glutamate in the ancestor of horse and cattle lysozymes, which requires only one base change to give glycine in the horse and none to retain glutamate in cattle. But this implies that the appearance of glutamate in the ancestor of the horse and cattle was not an adaptation to foregut fermentation, and makes it difficult to argue simultaneously that it was adaptive in the langur. In any case, the worse-than-random performance of methods based on the genetic code in cases where they can be checked⁴ suggests that it is safer simply to count amino-acid differences.

At first glance, the six sequences appear to contain abundant data that led to the initial observation that an unexpected tree showing the langur and cattle as close relatives required as few substitutions as the biologically reasonable tree. In reality, however, this result was derived from data at very few loci. Of 130 loci altogether, 47 show no variation and are not used by any method of tree construction. A further 63 show variations of the kind where any amino acid other than the main one occurs once only. Such variations contribute to tree-construction methods such as UPGMA⁵ that are based on the table of differences, but not to minimum-length ('parsimony') methods if no assumptions are made about the likely evolutionary route from one amino acid to another⁶. Only 20 lead to variations in the lengths of the possible trees linking the 6 sequences, and 7 of these (loci 23, 37, 41, 88, 90, 117 and 119) do not discriminate between the two trees of particular interest. Even considering only those loci that are ignored by the minimum-length approach, UPGMA generates a tree that relates the langur and baboon sequences closely to one another, less closely to the human sequence, and more distantly to the others.

ATHEL CORNISH-BOWDEN
Centre de Biochimie et de Biologie
Moléculaire,
CNRS, 31 chemin Joseph-Aiguier, BP 71,
13402 Marseille Cedex 9, France

STEWART ET AL. REPLY — We do not agree with Cornish-Bowden's statistical criticism. Our lysozyme sequences¹ were not sampled at random as he assumes. Rather, we chose representatives from two lineages that convergently lost an old function while acquiring the same set of new functions. We also required that the times when the new functions arose be recent enough to avoid the problem of multiple hits at the same amino-acid site, yet old enough to let a significant number

of amino-acid replacements accumulate. The lineages leading to langur and cow stomach lysozymes meet these requirements and they were the first functionally convergent pair to be analysed explicitly with the approach we described¹. Thus, there are no empirical grounds for regarding this set of lysozyme sequences as the tail of a distribution based on numerous other protein sets, each containing a pair of functionally convergent sequences that exhibit no convergence in primary sequence. For these reasons, we continue to consider stomach lysozymes to provide the most plausible case available for convergent evolution of protein primary structures in response to known new selection pressures impinging at known times; nevertheless, we admit that the case is not yet conclusive.

Cornish-Bowden raises two other points that reflect his unwillingness to adopt methods of analysing evolutionary history and his preference of distance methods. This reticence may stem in part from his attempt to analyse variation at position 50 in six mammalian lysozymes; if his analysis had included the other lysozymes of known sequence, as ours did¹, it would have been clear that convergence is the simplest explanation for the uniquely shared residues at this position. His preference for UPGMA is misguided because this method does not generate evolutionary trees; it is a purely phenetic method, which merely summarizes the distances between the sequences⁵. The parsimony method we used represents a genealogical analysis that takes account of the nature and locations of the sequence differences and of variation in evolutionary rates among lineages. It is only through parsimony analysis that parallel and convergent events can be distinguished from divergent ones. If investigators were to follow Cornish-Bowden's recommendation "simply to count amino-acid differences" thus precluding genealogical analysis, their ability to study the evolutionary process would be severely limited.

CARO-BETH STEWART
Hormone Research Institute,
University of California at San Francisco,
San Francisco, California 94143, USA

JAMES W. SCHILLING
California Biotechnology, Inc.,
Mountain View, California 94043, USA

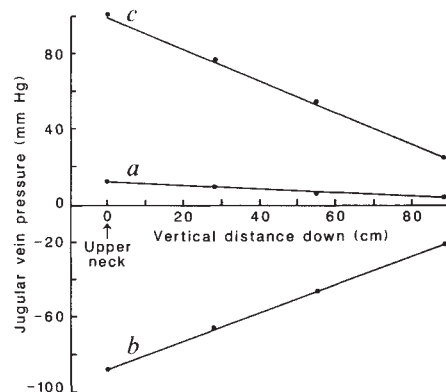
ALLAN C. WILSON
University of California at Berkeley,
Berkeley, California 94720, USA

1. Stewart, C.-B., Schilling, J.W. & Wilson, A.C. *Nature* **330**, 401-404 (1987).
2. Naithan, V.K. & Dhar, M.M. *Biochem. biophys. Res. Commun.* **29**, 368-372 (1967).
3. Gutte, B., Moser, R., Klausner, S. & Weilenmann, M. in *Dynamics of Biochemical Systems* (eds Ricard, J. & Cornish-Bowden, A.) 259-272 (Plenum, New York, 1984).
4. Kimura, M. *The Neutral Theory of Molecular Evolution* (Cambridge University Press, 1983).
5. Sneath, P.H.A. & Sokal, R.R. *Numerical Taxonomy* (Freeman, San Francisco, 1973).
6. Cornish-Bowden, A. *J. theor. Biol.* **101**, 317-319 (1983).
7. Stewart, C.-B. & Wilson, A.C. *Cold Spring Harb. Symp. quant. Biol.* **52**, 891-899 (1987).

Haemodynamics of the jugular vein in the giraffe

SIR—Controversy continues over the haemodynamics of the circulation to and from the head of the giraffe. The recent study by Hargens *et al.*¹ provides new information explaining the absence of oedema in the legs of the ambulant giraffe. But in sedated, standing giraffes the pressure gradient down the jugular vein is about one-tenth of, and in the opposite direction to, that expected for a standing column of blood. Hargens *et al.* suggest that compartmentalization of the blood in the vein is involved. This seems unlikely as the valves in the vein open towards the heart.

There is a more plausible explanation. The intravascular pressure at any point in the vascular system is the sum of two distinct pressures: (1) viscous flow pres-



Plot of pressure components in the jugular vein of the standing, sedated giraffe based on data from ref. 1. Note the increase in the gravitational component of pressure down the vein (*b*) and the decrease in pressure due to viscous resistance to flow (*c*). The actual pressure existing in the vein, line *a*, is equal to the sum of viscous flow and gravitational pressures. Because the drop in viscous flow pressure is greater than the rise of gravitational pressure, the actual pressure gradient down the vein shows a slight decrease.

sure; and (2) gravitational (hydrostatic) pressure. This distinction is important in haemodynamics and is often not sufficiently appreciated. Hargens *et al.*¹ measured the pressure down the jugular vein in three standing, sedated giraffes using the Millar Mikrotip transducer, which registers the pressure existing at the tip of the catheter. These pressures are shown in line *a* in the figure, which shows a fall from upper neck (+13 mm Hg) to lower neck (+4 mm Hg). Next, the gravitational pressure of the blood is plotted on the basis of the vertical distance in the jugular vein taken from the figure of these investigators (line *b*). Because the right atrial pressure is about zero (atmospheric), the gravitational pressure above the level of the heart is negative and increases from the head down.

From these two pressures one can

1. Hargens, A. R., Millard, R. W., Pettersson, K. & Johansen, K. *Nature* **329**, 59–60 (1987).
2. Pedley, T. J. *Nature* **329**, 13–14 (1987).
3. Goetz, R. H. *et al. Circ. Res.* **8**, 1049–1058 (1960).
4. Katz, A. I., Chen, Y. & Moreno, A. H. *Biophys. J.* **9**, 1261–1279 (1969).

1. Hogle, J.M. *Nature* **332**, 13-14 (1988).
2. Burke, K.L., Dunn, G., Ferguson, M., Minor, P.D. & Almond, J.W. *Nature* **332**, 81-82 (1988).
3. Arya, S.C. *Ann. Inst. Pasteur Virol.* **138**, 527-528 (1987).

Environmental sex determination in reptiles

SIR—Interest in environmental sex determination (ESD), particularly in reptiles, is growing, shown, for example, by the discussion in ref. 1.

Perhaps the most exciting speculations have centred on the phylogeny of sex-determining mechanisms in reptiles. The consensus appears to be that presence of genotypic sex determination in reptilian groups has evolved recently¹⁻³, with ESD being the plesiomorphic condition. This suggestion has prompted speculation concerning the cause of the extinction of dinosaurs at the end of the Cretaceous. As the story goes, dinosaurs possessed ESD and, when global temperatures dropped, offspring of only one sex were produced, effectively extinguishing the species^{1,4,5}. Yet there is a disturbing aspect to this model. If changes in global temperatures selectively extinguished dinosaurs because they possessed ESD, then how can the persistence of many extant reptilian species which possess ESD be explained? Turtle, lizard and crocodilian species all crossed the Cretaceous–Tertiary boundary without showing significant extinction⁶. Further, the disappearance of viviparous dinosaurs (such as ichthyosaurs) that vanished before the end of the Cretaceous, and which probably had genotypic sex determination, must be explained.

In all likelihood, ESD did evolve during ancient times, but perhaps under conditions differing from those that exist today. Thus, attempts to ascertain the 'advantages' of ESD for extant reptiles could be misleading. Persistence of ESD in many reptilian species may not be adaptive; its presence merely may not be maladaptive. We suggest that biologists interested in mechanisms of sex determination in reptiles undertake carefully designed studies to address such mechanisms, rather than muddy the waters of empiricism with unfounded speculation.

Those species with genotypic sex determination may be the animals on which attention should be focused, because it seems more likely that their systems have evolved recently and for good reason.

FREDRIC J. JANZEN

Department of Biology,
University of Chicago,
Illinois 60637, USA

GARY L. PAUKSTIS

NEC America Inc.,
Data Communications,
1255 Michael Drive, Wood Dale,
Illinois 60191, USA

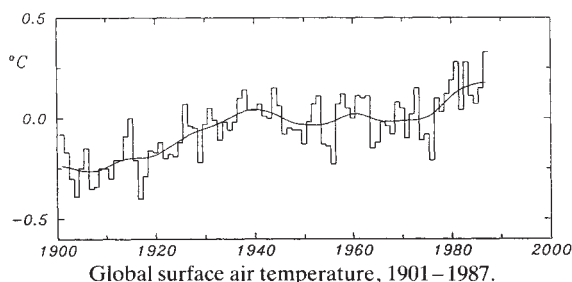
1. Head, G., May, R.M. & Pendleton, L. *Nature* **329**, 198–199 (1987).
2. Bull, J. J. *Q. Rev. Biol.* **55**, 3–21 (1980).
3. Bull, J. J. *Evolution of Sex Determining Mechanisms* (Benjamin/Cummings, Menlo Park, 1983).
4. Ferguson, M. W. J. & Joenen, T. *Nature* **296**, 850 (1982).
5. Standora, E. A. & Spotila, J. R. *Copeia* **1985**, 711 (1985).
6. Carroll, R. L. *Vertebrate Paleontology and Evolution* (Freeman, New York, 1988).

Evidence for global warming in the past decade

SIR—The global-mean surface air temperature is the most common measure of the state of the climate system. Variations of this parameter are probably determined largely by the sensitivity of the climate system to external forcing factors such as solar output, explosive volcanic eruptions and the changes in concentration of CO₂ and other radiatively active gases. Understanding the climate's response to changes in forcing is essential if we are ever to forecast future climatic change.

Increases of CO₂ and other radiatively active trace gases (methane, chlorofluorocarbons, nitrous oxide and tropospheric ozone) are expected to raise global equilibrium general circulation models¹ for a doubling of atmospheric CO₂. Up to now, the rise in equivalent CO₂ concentration (incorporating the radiative effects of the other trace gases) has lifted levels about 40% above their preindustrial level of around 280 p.p.m.v. (ref. 2).

In 1986, some of us reported³ the first



Global surface air temperature, 1901–1987.

compilation of global mean temperature estimates, spanning the period 1861–1984. We have now extended this analysis to include 1985–87 and to improve the ocean and land coverage for the 1980s. The improved coverage for the 1980s slightly alters the original values for 1980–84. Our new estimates use the comprehensive ocean atmosphere data set (COADS) sea-surface temperature (SST) data for 1980–86. For the marine regions for 1987, we used SST data from the UK Meteorological Office. SST data are a

1. Report of the International Conference on the Assessment of the role of carbon dioxide and of other greenhouse gases in climate variations and associated impacts, World Meteorological Organization No. 661 (Geneva, 1986).
2. Wigley, T.M.L. *Geophys. Res. Lett.* **14**, 1135 (1987).
3. Jones, P.D., Wigley, T.M.L. & Wright, P.B. *Nature* **322**, 430–434 (1986).
4. Folland, C.K., Parker, D.E. & Kates, F.E. *Nature* **310**, 670–673 (1984).
5. Jones, P.D. *et al. J. clim. appl. Meteorol.* **25**, 161 (1986).
6. Jones, P.D., Raper, S.C.B. & Wigley, T.M.L. *J. clim. appl. Meteorol.* **25**, 1213–1230 (1986).
7. Jones, P.D. *J. clim.* (in the press).
8. Hansen, J.E. & Lebedeff, S. *J. geophys. Res.* **92**, 13345–13372 (1987).
9. Wigley, T.M.L., Angell, J.K. & Jones, P.D. in *Detecting the climatic effects of increasing carbon dioxide* (eds MacCracken, M.C. & Luther, F.M.) 55–90 (US Dept of Energy, 1985).
10. Pan, Y.-H. and Oort, A.H. *Mon. Weath. Rev.* **111**, 1244–1258 (1983).
11. Yasunari, T. *J. met. Soc. Japan* **65**, 67–80 (1987).
12. Heath, D.F. *Nature* **332**, 219–227 (1988).
13. Kiehl, J.T., Boville, B.A. & Briegleb, B.P. *Nature* **332**, 501–504 (1988).

good substitute for air temperature over the oceans⁴. These two sources of SST data are in excellent agreement during the 1980s. We have also updated the land temperature series for the two hemispheres⁵⁻⁷.

The time series for the land and marine areas for 1901–87 is shown in the figure. 1987 is the warmest year recorded, 0.05 °C above the next warmest, 1981 and 1983. The warmth of the 1980s is most evident in the Southern Hemisphere. Here, seven of the eight warmest years have occurred during the 1980s, with 1987 being the warmest. Warmth over the Northern Hemisphere in the 1980s is not so startling, yet the two warmest years are 1981 (the warmest) and 1987.

The global land air temperature in 1987 is about the same as the previous maximum in 1981 (updating ref. 8), the difference in the analysed temperatures for these years being less than the uncertainty caused by incomplete station coverage. The warmth is not just a surface phenomenon. The global radiosonde data network⁹, which began in 1958, also confirms the warmth of the 1980s both at the surface and in the lower troposphere, particularly in 1981, 1983 and 1987. The network also provides data for the stratosphere, a region that models suggest should become increasingly cold as CO₂ levels increase. For global mean lower-stratospheric temperatures, the coldest years are 1985, 1986 and 1987.

It is likely that the record warmth in 1987 partly resulted from the strong 1986–87 El Niño/Southern Oscillation (ENSO) event^{10,11}, although this event was not as intense as the one in 1982–83. It is also possible that the stratospheric coldness in recent years is partly associated with the recent ozone depletion^{12,13}. Nevertheless, the persistent surface and tropospheric warmth of the 1980s which, together with ENSO, gave the exceptional warmth of 1987 could indicate the consequences of increased concentrations of CO₂ and other radiatively active gases in the atmosphere.

P.D. JONES
T.M.L. WIGLEY

Climatic Research Unit,
University of East Anglia,
Norwich NR4 7TJ, UK

C.K. FOLLAND
D.E. PARKER

Meteorological Office,
Bracknell RG12 2SZ, UK

J.K. ANGELL

Air Resources Laboratory
NOAA Environmental Research Labs,
Silver Spring, Maryland 20910, USA

S. LEBEDEFF
J.E. HANSEN

NASA Goddard Space Flight Center,
New York, New York 10025, USA

Rita and the four Gs

M. F. Perutz

In Praise of Imperfection: My Life and Work. By Rita Levi-Montalcini. Translated by Luigi Attardi. *Sloan Foundation Science Series/Basic Books*: 1988. Pp. 220. \$18.95.

RITA Levi-Montalcini received the Nobel Prize for Physiology or Medicine in 1986, together with Stanley Cohen, for their discovery of growth factors, made 30 years earlier while working together at Washington University in St Louis. This book recalls Levi-Montalcini's life from her childhood in Turin to her return to Italy from the United States in 1963. Its title derives from a poem by Yeats:

*The intellect of man is forced to choose
Perfection of the life, or of the work,
And if it take the second must refuse
A heavenly mansion, raging in the
dark.*

Levi-Montalcini chose the work.

André Lwoff wrote that: "The scientist's art is first of all to find himself a good master", to which I should add "and next, to find himself a good problem". Levi-Montalcini found her problem in a laboratory rigged up in her bedroom during the Second World War, when Mussolini's antisemitic legislation, a cowardly copy of the German, forced Turin University to expel all Jews, including her professor of anatomy Giuseppe Levi. He was the master who had aroused her interest in the nervous system when she was an intern, worked with her in her improvised laboratory, and encouraged her for the rest of his long life. They set out to analyse how excision of as yet non-innervated limbs from early chick embryos affected development of motor cells in the spinal cord and of sensory cells in the dorsal root ganglia, and were led to suspect that the limbs release a tropic factor that is conveyed by the growing axons to their cell bodies. After the war their publication came to the attention of Viktor Hamburger, a German émigré and pupil of the great embryologist Hans Spemann. Hamburger invited Levi-Montalcini to spend a semester with him at St Louis. That semester was to become 16 years.

The decisive observations came in an experiment that illustrates the importance of the prepared mind. Elmer Bueker, a

former pupil of Hamburger, sent him an article which described how a mouse sarcoma, grafted on to a chick embryo, had become innervated by fibres from the embryo. Bueker concluded that the tumour had provided more ample terrain for the growth of the nerve fibres than



Wide-eyed awareness — Rita Levi-Montalcini in 1920.

the nearby embryonic limb, but Levi-Montalcini thought otherwise. In a euphoric mood, she dropped all current research in order to repeat Bueker's work. On being grafted to her embryos, the tumours Bueker had used became innervated as Bueker had described, but another tumour sent to her (by mistake?) produced a far more dramatic effect. It caused the non-innervated organs of the embryo, including its viscera and blood vessels, to be invaded by large bundles of nerve fibres. She writes:

This observation indicated that the tumour had released a humoral, a fluid, factor able both to accelerate differentiative processes in sympa-

thetic and, to a lesser degree, sensory cells; and to cause excessive production, as well as the quantitatively and qualitatively abnormal distribution of nerve fibres.

What was that factor?

Its nature revealed itself by a remarkable stroke of luck, after Stanley Cohen, then a young biochemist, had joined her at St Louis. In experiments carried out in Carlos Chagas and Hertha Meyer's laboratory at Rio de Janeiro, the author had established that extracts of mouse tumours induced the formation of haloes of nerve fibrils around cultured sensory ganglia, but only if the tumours had first been transplanted into and then excised from chick embryos. Levi-Montalcini and

Cohen spent a year trying to extract enough of the factor from such transplanted mouse sarcomas to characterize it, and thought it was a nucleoprotein. When Cohen wondered if the nucleic acid might be a contaminant, Arthur Kornberg, then at St Louis, suggested treating the extract with snake venom whose phosphodiesterase breaks down nucleic acid; instead of destroying the activity the snake venom raised it spectacularly. It turned out that it contained a several thousand times greater concentration of the growth factor than the mouse tumours. This discovery allowed Cohen to isolate and characterize the factor and provided Levi-Montalcini with pure factor to inject into her embryos. Had they been able to buy pure phosphodiesterase from Sigma, they would never have discovered this.

The first part of the book is an interesting documentary of life in a Jewish middle-class family in fascist Italy before and during the Second World War, especially during the Nazi terror, when the Levis were hidden in Florence under false names by good-natured, courageous gentiles who pretended not to know that they were Jews. A far greater proportion of Jews survived in Italy than in any other country of continental Europe, because gentiles helped and hid them, often at the risk of their lives.

Paul Ehrlich has said that success in research needs four Gs: Glück, Geduld, Geschick und Geld (luck, patience, skill and money). Levi-Montalcini's book shows that she had the first three in good measure and that she needed little of the fourth. □

M. F. Perutz is at the MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

Mathematics and the model

Peter E. Hodgson

The Interacting Boson Model. By F. Iachello and A. Arima. *Cambridge University Press*: 1987. Pp. 250. £32.50, \$59.50.

THE nuclear shell model has been spectacularly successful in providing a detailed account of the light nuclei and of heavier nuclei around doubly closed shells. Unfortunately it is quite impracticable to apply it to heavy nuclei away from closed shells because of the complexity of the configurations in shell model terms. Yet such nuclei do show simple structures, such as rotational and vibrational bands, that demand a simple explanation, and this has been provided by the collective models. It is, however, unsatisfactory to have different models for different nuclei; there are no sharp divisions between the types of nuclei. In the case of collective nuclei, what is needed is a way to truncate the full shell-model space so that it still contains most of the essential physics. The interacting boson model is one of the most successful attempts to do this.

The model originated in the work of Feshbach and Iachello, who in 1969 analysed light nuclei in terms of interacting bosons, and in that of Janssen, Jolos and Döna, who in 1974 proposed that collective quadrupole states can be described in terms of the SU(6) group. The model was further developed by Arima and Iachello, who realized that the bosons can be interpreted as nucleon pairs, thus providing a microscopic model of nuclear excitations. Initially the formulation was in terms of s and d bosons, and it was found that for even nuclei the Hamiltonian containing all possible boson interactions can be diagonalized exactly, giving an analytical expression for the energies of the excited states. Subsequently the model was extended to include transition probabilities, and was found to agree well with a wide range of experimental data.

A further elaboration was the introduction of unpaired fermions, thus bringing odd-even nuclei within the model's scope. Other refinements include configurational mixing and giant resonances, and the model has been applied successfully to light as well as to heavy nuclei.

This is the first book devoted to the interacting boson model, and as it is written by the two physicists who have been most prominent in developing the theory, it is destined to be a standard work on the subject. The authors' purpose is to present the basic mathematical framework as it is applied to even nuclei, and to collect together all the formulae that have been used over the years to account for the

collective properties of nuclei. A second volume is promised on even-odd nuclei, and then a third on the microscopic structure and justification of the model.

The first section of the book is devoted to the boson calculus, the mathematical technique fundamental to the theory involved. This has wide applications to other branches of physics such as molecular structure. The boson calculus is described in detail, and formulae are given for physical quantities such as electromagnetic transition rates, binding energies, radii and moments of inertia. The second section is concerned with the refinements that are necessary in order to

include in the model the distinction between neutrons and protons, and the third section considers other refinements such as the inclusion of isospin.

The treatment throughout is highly mathematical, with heavy reliance on group theory, and little attempt is made to describe the physical ideas underlying the model in a way that will be intelligible to experimentalists. But for theorists the book provides a systematic account of the subject that will be invaluable for easy reference. □

Peter E. Hodgson is Head of the Nuclear Physics Theoretical Group, Nuclear Physics Laboratory, University of Oxford, Oxford OX1 3RH, UK.

Relative reactions

C.W. Kilmister

The Comparative Reception of Relativity. Edited by Thomas F. Glick. *Reidel*: 1987. Pp. 412. Dfl. 198, £54.50, \$79.

Einstein in Spain. By Thomas F. Glick. *Princeton University Press*: 1988. Pp. 391. \$42, £23.50.

SPECIAL relativity shares with quantum mechanics the property of being in conflict with common sense. So in both cases the question of their reception is a complex one, involving the reactions of the scientific and the lay communities (who, whatever they may think, do not act independently of each other).

In 1983, a double session at a colloquium in Boston looked at the reception of relativity on a national basis, and was enough of a success to suggest a combined volume by some of the participants and other contributors. What is most interesting is not the way in which different countries eventually came to the same view of relativity as part of scientific orthodoxy, but the national differences, whether produced by political or cultural contexts.

In the United States, as Goldberg shows, the early emphasis was still on ether drift experiments. After that, the dominant concept of usefulness — either in applications or in the sense that any theory that was true had to be comprehensible to everyone — required that the new theory should be fitted into an epistemological framework that was already familiar. The ether also played an important part in Britain, according to Sanchez-Ron's chapter, but in a more theoretical way, for the heritage of Larmor (to go back no farther) needed the new theory to be made compatible with some kind of ether. But, in Britain, there were other important currents; Cunningham was very clear about the role of the Lorentz transformations in Maxwell theory by 1907, Robb gave his new axiomatization in 1911 and, although

Jeans was slow to admit relativistic ideas to his *Electricity and Magnetism*, his final revisions in 1920 showed a complete understanding of them.

The emphasis in Germany was almost as opposed to that in the United States as it is possible to imagine. Pyenson sees the touchstone to acceptance there as the revolutionary character of the theory. Politically suppressed intellectuals sought a freedom in the realm of ideas that was denied to them elsewhere; but also the need was felt for a new vision to synthesize the specialist knowledge into a unified view of the world. Pyenson works out a detailed analogy, character by character, with the French Revolution, but this chapter, perhaps the best in the book, contains much else besides.

In France itself, on the other hand, notwithstanding the contributions of Poincaré and Langevin, the theory made little progress for many years, and only in the 1950s did textbooks and university courses reflect its acceptance. The great tradition of mathematical physics acted as a barrier to progress in any field that needed to cross the divide between physics and mathematics. So much is clear from Paty's chapter, but the point is further emphasized by Biezunski's account of Einstein's visit to Paris in 1922. Here, a momentary change of attitude seemed to overcome the disadvantages in French eyes of the theory being revolutionary and of German (indeed Jewish) origin, but the potential generated by the visit was never realized.

In Italy, according to Reeves, not only was Einstein's use of the glories of Italian differential geometry appreciated, but Mussolini himself gave public approval to the theory as early as 1921. No matter that this was a vulgar confusion between relativity and relativism; the practical help was there. Spain, according to Glick, followed Italy, but he deals with this in greater detail in the other book under review, which I mention below.

Vizgin and Gorelik give a detailed account of the reception of the special and general theories in Russia and the USSR;

here the break between the two fits nicely onto the political divide. Before 1914, in an underdeveloped country with most of the research centred on Moscow and Petersburg, there was interest in the special theory but a good deal of etherial thinking in the British mould. But the general theory came in 1919 to a new country in an intellectual ferment. The story of the struggles in the 1920s and later about whether the theory was consistent

From Einstein in Spain



Special influence — Einstein in Toledo, 1923.

with Marxism is spelled out, though without much detail about the role of the Party in compelling consent. Finally, the chapters by Średniawa and Kaneko on Poland and Japan describe two isolated cases.

The book as a whole is a worthy attempt to survey a large field. It is not, it is true, all that exciting, but every chapter contains a full and useful bibliography.

Nineteenth-century Spain, as described in *Einstein in Spain*, had, from 1857 onwards, a centralized and doctrinaire educational system in which the ministry had dictatorial powers over curriculum and textbooks and could remove professors at will for espousing 'erroneous' doctrines of a moral, religious or political kind. The inevitable breach of the dam came in 1900, so in time for the advent of the special theory, which was backed by both the right and the left in that highly polarized community.

The political and cultural background is fully dealt with in Glick's account of how Einstein came to be invited to Spain in 1923 and what the consequences were for many questions, such as the debate over the role that science should play in Spanish society. The story is a fascinating one, well told. □

C.W. Kilmister, 11 Vanburgh Hill, Blackheath, London SE3 7UE, UK, was formerly Professor of Mathematics at King's College, London.

Japanese finesse

R. W. Cahn

Fine Ceramics. Edited by Shinroku Saito. Elsevier: 1988. Pp.352. \$59.95, £44.

ADVANCED ceramics, new ceramics, high-tech ceramics — these are a few of the terms used in the West to denote what the Japanese prefer to call 'fine ceramics'. These materials are not necessarily of complex composition — they include Al_2O_3 and SiC , among others — but they all share certain characteristics: they are used for purposes for which no ceramics at all were used a few decades ago; to give satisfactory service, they must be processed according to precisely controlled, often elaborate schedules; and their exact compositions and processing must be optimized by lengthy, detailed and intellectually demanding research.

The Japanese have chosen fine ceramics as one of their preferred high-technology areas, and this volume was created to give an account of their achievements to date. The Japanese edition appeared in 1985 and has now been translated into excellent English (by an unnamed translator). Clearly no revision was undertaken for the translation, because no references later than 1985 are cited and there are bibliographical entries such as "1984, in press". Even so, the book is only slightly dated.

In his illuminating introduction, Saito explains that Japanese research on fine ceramics was greatly stimulated by the Ministry of International Trade and Industry (MITI) Project for Future Industrial Research, which was started in 1982 partly to "counter criticism from abroad that Japan has always received a 'free ride' on foreign technology". He emphasizes that MITI decided to limit its support "to projects too risky for private companies to carry out R&D, and to immature markets". Hence, fine ceramics for electronic and electrical applications, by far the largest market sector, were left to industrially financed research, and MITI concentrated its support on structural ceramics, materials for advanced internal combustion engines in particular.

The book contains articles on 39 distinct topics, grouped under 4 main headings: ceramic processing, characterization, structural ceramics and electronic ceramics. Forty five authors have contributed: of these, 16 work in industry, 23 in universities (there is one joint appointment) and 7 in government laboratories.

The primacy accorded to processing is characteristic of the Japanese approach: in fact, throughout the book authors return again and again to the processing needs of various novel ceramics, and in the particularly demanding field of structural ceramics, processing is the over-

riding theme. As it is here conceived, processing presupposes a subtle understanding of solid-state chemistry, which is a major feature of several articles.

The bibliographies are predominantly Japanese; Western literature is cited only insofar as it is necessary to set the scene. In view of the declared objective of the book, which is intended to be a "greeting from Japan to colleagues in the ceramic industry worldwide", this is as it should be.

Numerous Japanese technological and scientific innovations are summarized, especially in the electronic field: Furuhashi and Toda's very full survey of ferroelectric and electrooptic materials is an especially fecund source of information. Ura on the Japanese innovation of BeO -doped SiC to replace Al_2O_3 for ceramic packaging of integrated circuits is also outstanding; it is no wonder that Japan has cornered the world market in this speciality. Shirasaki and Kakegawa's study of the role of Ba^{2+} vacancies in governing an insulating-semiconducting transition in La -doped BaTiO_3 is a fascinating scientific detective story.

Particular interest attaches to the articles on structural ceramics, which reveal a record of painstaking and persistent testing of components in relation to numerous processing variables. On reading these articles, I have fully appreciated for the first time why Japan is steadily taking the lead in this risky field also. From a commercial viewpoint, the remark thrown out by the editor that Japan, alone in the world, has begun to manufacture household merchandise such as knives and scissors from ceramics, is evidence of a carefully thought-out piece of 'contrarian investment'.

Because of its various asides on policy making (by MITI, by a new ceramic research association, and by the Ministry of Education, Science and Culture, which in 1982 initiated an *academic* cooperative project on 'functional' — that is, electronic — ceramics), the book should be of value to research directors as well as to research ceramists. Generally, the close symbiosis between government, industry and academe emerges at various points throughout the volume.

As a systematic source-book, *Fine Ceramics* is unequalled. It warrants close attention from a wide readership.

R.W. Cahn is in the Department of Materials Science and Metallurgy, University of Cambridge, Pembroke Street, Cambridge CB2 3QZ, UK.

• Longman have just issued a paperback edition of *Physics of Amorphous Materials* by S.R. Elliott. The book is an introduction to non-crystalline materials for students of physics, physical chemistry, electrical engineering and materials science, and also a reference source for those in industry. Price is £15.95. For review see *Nature* 314, 26 (1985).

Bafflers, binders and coral relations

Anthony Hallam

The Evolution of Reef Communities. By J. A. Fagerstrom. Wiley: 1987. Pp. 600. \$74.95, £65.

OVER 30 years ago the distinguished American oceanographer Roger Revelle wrote that "of all earth's phenomena, coral reefs seem best calculated to excite a sense of wonder", a remark that Charles Darwin would probably have endorsed. Since that time our knowledge of them has expanded enormously, and the question has naturally arisen for palaeontologists and geologists as to how good a model modern coral reefs are for the ancient reefs in the rock record. Fagerstrom's book is the first comprehensive attempt to compare reefs ancient and modern from a variety of biological and geological aspects, and to attempt an interpretation of reef community evolution.

Reefs are mainly biological phenomena. They are characterized by structural rigidity and topographic relief produced by the rapid growth of large colonial or gregarious skeletal organisms living in close proximity to each other, and (compared with adjacent non-reef areas) by their high taxonomic diversity; they are *not* characterized either by water depth or geological location. A bewildering variety of organisms make up the reef communities, including algae, sponges, coelenterates, bryozoans, molluscs, brachiopods and crinoids. As the taxonomic composition has changed significantly through time, there is a need to generate another kind of classification for purposes of effective comparison. This Fagerstrom does by proposing an ecological classification of five guilds, termed constructor, baffler, binder, destroyer and dweller.

Part 1 of the book is devoted to the development of a series of models for Cenozoic reefs, Part II to an account of the autecology of the main reef-building taxa, and Part III to the synecology and history of pre-Cenozoic reef communities, from the late Precambrian to the Cretaceous. Fagerstrom argues that the Cenozoic, more particularly Holocene, photic-zone reefs, dominated as they were by rapidly growing members of the constructor guild, the zooxanthellate scleractinian corals, provide a very unsatisfactory ecological model for older reefs, especially the late Palaeozoic ones dominated by members of the baffler and binder guilds, notably algae, sponges and bryozoans. By the late Triassic, scleractinians became of major importance for the first time, but in the Cretaceous they became subordinate to rudistid bivalves.

Cenozoic models are far more adequate

and useful for recognizing and interpreting the predominantly non-biological aspects of earlier times, involving the destruction, transport and deposition of calcareous sediment. Nevertheless comparison of changing guild structure provides an illuminating means of comparing reef communities through time. Fagerstrom concludes that these communities have exhibited long periods of surprising stability punctuated by shorter episodes of more rapid change resulting from mass extinctions and macroevolutionary events.

The style of writing leaves little to be desired, being lucid and comparatively free of jargon. Descriptions are satisfyingly thorough, arguments well presented and interpretations generally well balanced and reasonable, while the overall organization is sensible and user-friendly. The subject matter is comprehensive rather than exhaustive, with important examples such as the spectacular Devonian reefs of Morocco being omitted, but my only serious complaint about an otherwise

admirable book concerns the photographs. Rather than being interspersed with the text, where they would have been most useful, they are grouped together at the end with the stratigraphic horizons and locations divorced from the captions and put together on separate pages.

Even more frustrating, however, is their poor quality and lack of labelling, so that it is often difficult to perceive anything distinctive. Surely the grouping together of photographs should have allowed the insertion of glossy paper to give much higher quality of reproduction? □

Anthony Hallam is Lapworth Professor of Geology in the University of Birmingham, Birmingham B15 2TT, UK.

• Also recently published by Wiley is *Coral Reef Geomorphology* by André Guilcher. The book is primarily geomorphological rather than biological in content, dealing with the shape and types of reefs, their structure, damage caused by human activity, and the requirements for reef protection and recovery. Price is £29.50, \$67.95.

Soft engineering

A. Serafini-Fracassini

Biological Materials: Structure, Mechanical Properties, and Modeling of Soft Tissues. By Frederick H. Silver. New York University Press: 1987. Pp.229. \$42.50.

G. H. MEYER's classical paper, dealing with the correlation between the disposition of the systems of platelets in the spongiosa of the metatarsal head and the pattern of the lines of maximal pressure and tension, was published in 1867. Since then, two developments have had a profound effect on our understanding of the structure-function relationship of stress-bearing animal tissues.

The first was the acquisition of a detailed knowledge of the chemical structure of two classes of extracellular biopolymers, collagen and elastin; these are capable of determining the mechanical properties of tissues by the provision of elastic components that cover a wide range of values for the Young's modulus. The second development was the discovery of elaborately textured ultrastructural interactions of collagen and elastin with extracellular glycoprotein and proteoglycans. Such interactions lead to the formation of architectural patterns, on a scale from the macromolecular to the macroscopic, which respond effectively and economically to intrinsic and extrinsic stimuli, and which exhibit a high degree of mechanical appropriateness.

In this monograph, Dr Silver skilfully presents an integrated view of the structure and the engineering properties of soft connective tissues, with useful appraisals

of recent developments in this field and concise but clear surveys of fundamental technologies. The book is not intended for specialists. Rather, it will meet the needs of students whose interest lies in the use of biomaterials and who want to acquire a sound reading knowledge of biomechanical engineering.

Chapters 1, 2 and 3 provide short introductions to the nature of the different classes of biomaterials, the chemistry of the constituent macromolecules, and the structure of connective and cardiovascular tissues. Chapter 4 deals with the gross conformational analysis of connective tissue biopolymers in solution, and introduces techniques suitable for the estimation of their size and shape and for the evaluation of their frictional properties. The self-assembly of collagen is discussed thoroughly in Chapter 5.

In the remainder of the book, Silver develops the analysis of the mechanical properties of tendon, skin, aorta and articular cartilage. Moreover, he attempts to set out, in as elementary a form as possible, the way in which stress-strain relationships can be idealized to provide a basis for a mathematical theory suitable for biomechanical modelling.

The book's usefulness is somewhat reduced by the unduly narrow focus on collagen. Also, little or no attention is given to such important topics as the molecular theory of elastic recoil and the generation of osmotic pressure in cartilage by steric macromolecular exclusion and Donnan effect. But overall Dr Silver has written a volume that will be a valuable addition to the literature. □

A. Serafini-Fracassini is a Professor in the Department of Biochemistry, University of St Andrews, St Andrews, Fife KY16 9AJ, UK.

Who's paying for new drugs?

Robert K. Oldham

Direct payment by patients for treatment with drugs still in development is the subject of controversy. The matter goes deeper than it might first appear.

DRUG development is expensive and slow. Each new anti-cancer drug, for example, comes to the practising oncologist in the United States at a cost of \$60–\$100 million and after a period of 8–12 years of research. The major portion of costs and time is taken up by clinical trials designed to demonstrate the drug's effectiveness and acceptable toxicity to federal regulatory authorities.

The classic pattern of drug development requires thorough studies *in vitro* and in animal models, followed by Phase I clinical studies on human beings to delineate toxicities. Phase II studies are then conducted to determine the drug's efficacy. Given acceptable toxicity and some evidence of anti-cancer activity, Phase III trials are carried out to demonstrate a benefit in patient survival or quality of life, and to compare the new drug with existing treatments. This has been the process of drug development for over 30 years, and it is uniformly applied to anti-cancer (and most other) drugs by the Food and Drug Administration (FDA), pharmaceutical companies, and government and university researchers.

Over the past three years, my own company has been criticized for accepting patients' money for laboratory research services leading to experimental treatments. But it is pertinent to ask who, elsewhere in the clinical research system, is footing the bill for trials with new drugs.

Grants and contracts

Historically, pharmaceutical companies and the government awarded grants and contracts to universities for the conduct of clinical trials. The financial support for these trials came from profits accumulated from the sale of pharmaceutical products (pharmaceutical companies) and funds from taxpayers (government-sponsored trials). Obviously, the former funds originally belonged to patients and the latter were paid by patients and taxpayers. But hitherto a direct link between the patient and the funding of clinical trials research has not been apparent.

As the National Cancer Institute (NCI) has built up its network of Cancer Centers, Community Hospital Oncology Programs and Community Clinical Oncology Programs, however, the connection between drug development and patients paying for research has become more obvious. Phase I and II trials are still

largely conducted by universities and, in theory, the funding for these trials comes from pharmaceutical company grants and contracts, as well as taxpayers' dollars. But even for these early trials (for example of interleukin-2/activated killer cells), there has recently been a closer linkage with patient funding. Patients and their insurance companies are being billed for hospital and laboratory costs, and sometimes for professional fees. These clinical research protocols are uniformly peer reviewed and approved with the investigational agents provided by the NCI and/or the sponsoring pharmaceutical company. All require FDA registration, because the research is being carried out under an investigational new drug (IND) application cleared by the FDA.

It is clear that insurance companies are increasingly being charged for such trials through standard hospital and clinic billing systems. There has been a great deal of debate on the Diagnosis Related Group (DRG) system as it relates to research funding. The lack of a research DRG has made more apparent the lack of access to experimental medicine of those depending on Medicare. Private insurance companies continue to be billed for clinical research, however, sometimes without the insurance company being informed that the patient was being treated under an experimental protocol.

Phase III trials are even more closely connected to the practice of medicine and the standard method of insurance reimbursement in the United States. For experimental trials of anti-cancer drugs, the protocols are often conducted in cancer centres and as part of NCI clinical cooperative groups. These cancer centres and cooperative groups derive a large portion of their funding from the NCI and their protocols are reviewed and approved within the NCI system.

The Phase III protocols often involve testing two chemotherapeutic drugs against three others, or one sequence of administration against another. The drugs may be investigational, with testing under an IND, or they may be new combinations of approved drugs being used experimentally in sequences or combinations not yet approved for routine use. Because drugs are approved by the FDA for other indications, and because the studies of toxicity and efficacy are often based on trials conducted with the drug as a single agent, new

combinations and sequences represent the experimental use of drugs even when the agents are commercially available.

Experimental protocols

Theoretically, these combinations and sequences should have Phase I (toxicity) and Phase II (efficacy) testing before they are put into widespread use. But this has not been the case. Physicians, pharmaceutical companies and the NCI routinely conduct Phase III trials of new combinations and novel sequences as experimental protocols; these protocols are carried out as clinical research and published in medical journals. Although there is some taxpayer support for such trials through NCI cancer centres and cooperative group programmes, the bulk of the hospital, clinic and laboratory costs is paid for by insurance companies and the patients. Insurance payment for experimental medicine represents patient-funded research, and the difference between the insurance reimbursement and the billed amount is often paid by the patient. Thus, the main source of funding for Phase III trials such as these is the patient.

When costs are paid directly by the patient, the patient-funded research linkage is clear for all to see. When an insurance premium is paid and insurance reimbursement covers these costs, it is still patient-funded research with a somewhat less clear linkage. Similarly, when a pharmaceutical company uses research and development funds derived from the sale of approved drugs to pay for research trials with new products, the money actually comes from patients paying for approved products and is thus patient-funded research. This is yesterday's patient paying for tomorrow's patient's research. The linkage is here one step further removed from obvious patient-funded research. Finally, when taxpayer dollars are used for research, the funding is coming from a mixture of individuals most of whom are not yet patients.

Is it acceptable for patients to fund research indirectly but not acceptable when the direct relationship is apparent? The question of who pays for clinical research needs broad public debate and openness on the part of all the parties involved in developmental therapeutics. □

Robert K. Oldham is Director of the Biological Therapy Institute, Hospital Drive, Franklin, Tennessee 37064, USA.

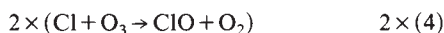
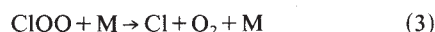
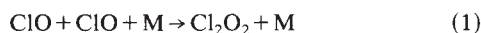
The stability and photochemistry of dimers of the ClO radical and implications for Antarctic ozone depletion

R. A. Cox & G. D. Hayman

Engineering Sciences Division, Harwell Laboratory, Didcot, Oxon OX11 0RA, UK

Measurements of the ultraviolet spectrum of the Cl₂O₂ molecule formed at temperatures in the range 203–300 K by recombination of ClO radicals are reported. The equilibrium constant for the reaction has been determined and the products of photolysis of Cl₂O₂ at 254 nm investigated. The main photodissociation pathway for Cl₂O₂ probably produces Cl atoms and chlorine peroxy radicals, as assumed in calculation of the ozone loss by the ClO and Cl₂O₂ catalytic cycle in the Antarctic stratosphere.

THE reported findings^{1,2} of extraordinarily high concentrations of the ClO radical in the springtime Antarctic stratosphere support the theory that catalytic chemical destruction is an important factor in the large losses of ozone over Antarctica observed in recent years^{3,4}. The catalytically active radicals NO₂ (ref. 5) and BrO (ref. 6) have also been implicated in this phenomenon. NO₂ is only present at very low concentrations^{7,8}, and is unlikely to contribute significantly to ozone depletion. These findings point to an important role for catalytic cycles involving the reaction of BrO with ClO (ref. 6), and particularly for the self-reaction of ClO with photodissociation of the Cl₂O₂ molecule formed in the reaction⁹:



where M is any other molecule. Assessment of the rate at which ozone (O₃) can be destroyed by this reaction sequence in the polar stratosphere requires accurate knowledge of the rates of formation of Cl₂O₂ (1), photolysis (2), and also of competing reactions of Cl₂O₂, which may depend on the structure of the molecule. The symmetrical structure Cl–O–O–Cl would facilitate the formation of the unstable chlorine peroxy radical ClOO in reaction (2), whereas photolysis of the asymmetrical structure Cl–O–Cl–O could lead to formation of the stable chlorine dioxide molecule, OClO, together with a Cl atom. In the stratosphere, OClO will be rapidly photolysed to yield an oxygen atom, leading to regeneration of O₃



This would lead to no net O₃ removal from the ClO self-reaction cycle.

Very few experimental studies of Cl₂O₂ have been made and its structure and chemical properties are not well-established. We report measurements of the ultraviolet spectrum of Cl₂O₂ formed at low temperatures (203–303 K) by recombination of ClO radicals produced in the reactions of Cl atoms with Cl₂O and with O₃. Experiments were also performed to investigate the equilibrium constant for reaction (1) and the products of photolysis of Cl₂O₂ at 254 nm.

A simple static photolysis technique was used with photodiode array spectroscopy to investigate ClO dimer formation. The reactions were conducted in a cylindrical quartz cell, 120 cm long and 3.0 cm internal diameter, internally coated with halocarbon wax to reduce surface reactions. The cell tem-

perature was controlled at selected values in the range 200–300 K by circulation of ethanol from a cooler/circulator unit (Huber HS80) through a jacket surrounding the cell. Photolysis was by 120-cm long 40-W fluorescent blacklamps ($\lambda_{\text{max}} = 350$ nm) or UV lamps ($\lambda_{\text{max}} = 254$ nm) mounted radially around the cell in a sealed reflecting housing. A well-stabilized deuterium lamp provided the spectroscopic light that traversed the long axis of the cell before dispersion on a 0.5 m spectrograph (B & M Spectronik) and detection on a 1,024-channel photodiode array (Reticon), giving a spectral coverage of 82 nm. The detector was coupled to a multi-channel analyser (Tracor Northern TN 1710) which controlled the detector functions and provided on-line handling and storage of the data. Further details of the system have been described¹⁰.

The gas mixtures were introduced into the cell by flowing from a manifold at 1 atm pressure. The flow was stopped and the spectrum recorded on the diode array, referenced to the light transmitted through the empty cell. The photolysis lamps were then activated for the required period and sequential spectra recorded at chosen time intervals. Spectra were recorded both during and after photolysis, so that the behaviour of the system could be observed both with and without illumination. Cl₂ was taken from a cylinder containing a 5% Cl₂ mixture in N₂. Cl₂O was prepared by passing the mixture through a column of yellow HgO powder and OClO was made by passing it through NaClO₂ crystals. O₃ was produced by electrical discharge of pure O₂. Both N₂ and O₂ were used as diluent gases for the gas mixtures.

The spectra were analysed by scaled subtraction of reference spectra recorded for known reactants and products, that is, Cl₂, Cl₂O, O₃, OClO and ClO, put on an absolute basis using the best available values of the absorption cross-sections, taking into account temperature-dependence where appropriate. To facilitate the search for residual-product spectra, arising for example from Cl₂O₂, spectra were referenced to the pre-photolysis spectrum so that only changes in reactant and product absorptions were seen. Scaled addition of the precursor molecule for ClO then gave a measure of the amount of ClO and Cl₂O₂ generated, and provided a basis for determination of the absorption cross-section of product molecules by mass balance. The precursor decay could be compared with the independently measured photolysis rates of Cl₂, Cl₂O and OClO as a check on consistency.

Figure 1 shows residual spectra in the 220–300 nm range from systems in which three different sources of ClO were used. The first spectrum was obtained in the photolysis of Cl₂-O₃ mixtures at 263 K, when ClO is produced from reactions (7) and (4)



Scaled addition of O₃ was based on the characteristic discrete

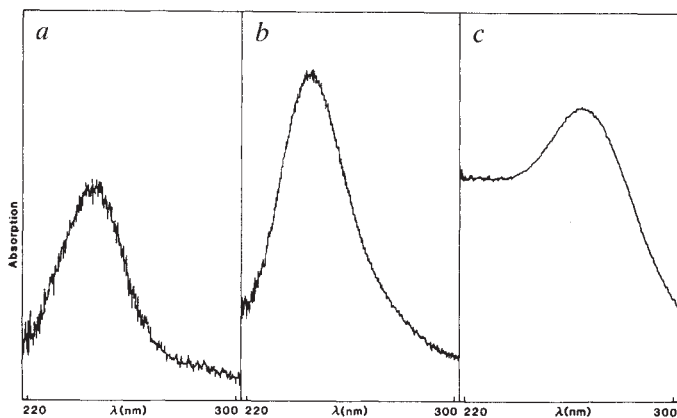


Fig. 1 *a*, Residual absorption from photolysis of $\text{Cl}_2/\text{O}_3/\text{N}_2$ mixtures. *b*, Residual absorption obtained from photolysis of $\text{Cl}_2/\text{Cl}_2\text{O}/\text{N}_2$ mixtures. *c*, Residual absorption obtained from photolysis of $\text{OCIO}/\text{N}_2/\text{O}_2$ mixtures.

O_3 absorption features near $\lambda = 260$ nm, and subtraction of ClO based on one or more of the discrete bands in the $A^2\Pi \leftarrow X^2\Pi$ system at $\lambda > 270$ nm.

The second spectrum was obtained in the photolysis mixtures of Cl_2 and Cl_2O at 233 K, when reactions (8) and (9) produce ClO



Scaled addition of Cl_2O was based on the removal of a negative-going residual at 270 nm and on the known rate of Cl -atom production from photolysis of Cl_2 and Cl_2O . Subtraction of ClO was made on the basis described above. Similar spectra were obtained from this system over a wide range of temperatures (200–303 K), reactant concentrations, and extents of reaction, both during and after photolysis.

The third residual spectrum resulted from the photolysis of OCIO in the presence of $\text{N}_2 + \text{O}_2$ diluent, the latter being added to scavenge O atoms produced in the photolysis:



The residual was obtained after scaled subtraction of OCIO based on the $a(19)$ band at 300.8 nm and subtraction of O_3 and ClO made on the same basis as above. A similar, but not identical, spectrum was found when O_2 was replaced by N_2 , or when Cl_2O was added to scavenge the O atom. These spectra were clearly different from those using the first two precursors and furthermore, the spectra with OCIO present showed changes in shape with time and temperature, indicative of secondary chemistry or the formation of metastable intermediates.

Our further studies have concentrated on the characterization of the residual absorption centred at 245 nm in Fig. 1*a* and *b*, which appeared to represent the main stable product of the ClO recombination at temperatures below 273 K. The time-dependence of the different molecules present in the static photolysis of $\text{Cl}_2 + \text{Cl}_2\text{O}$ mixtures at 265 K is shown in Fig. 2. During photolysis Cl_2O was removed at a constant rate, with an accompanying growth of ClO and of the residual absorption assigned to Cl_2O_2 . OCIO production was very slow at first, but growth continued when photolysis stopped. Both ClO and the residual absorption decayed in the dark and a slow decay of Cl_2O was also noticeable. At higher temperatures all the loss reactions were more rapid, so that at $T > 288$ K the only products detected after 50 s reaction time (with 10 s photolysis) were Cl_2 and OCIO , and these were in good mass balance with the initial Cl_2O . At $T \leq 258$ K, only small amounts of ClO and OCIO were formed,

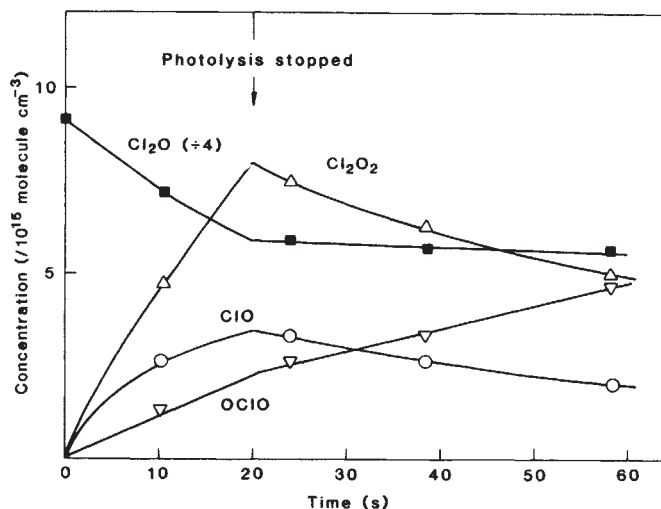
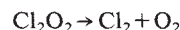


Fig. 2 Time-dependence of the species present in the photolysis of $\text{Cl}_2/\text{Cl}_2\text{O}/\text{N}_2$ mixtures of 265 K with 20 s photolysis.

and Cl_2O_2 and Cl_2O only decayed very slowly in the dark (half life, 1000 s at 233 K). This dark decay showed little temperature-dependence, and consequently was unlikely to arise from thermal decomposition of Cl_2O_2 in the gas phase, either by bond fission or by a four-centre reaction forming Cl_2 and O_2 :



The dark decay of Cl_2O_2 and Cl_2O at low temperatures could be due to loss by adsorption and decomposition on the surface of the reaction vessel, or to scavenging by Cl atoms from an unidentified source.

These behaviour patterns can be understood in terms of a mechanism involving production of ClO from reactions (8) and (9), followed by the fast recombination reaction ($k_1 = 6.0 \times 10^{-32} \text{ cm}^6 \text{ molecule}^{-2} \text{ s}^{-1}$ at 300 K (ref. 11), to form the dimer responsible for the residual absorption. The instability of the dimer at temperatures > 280 K and at the relatively high ClO concentration present under these conditions suggests that back-decomposition of Cl_2O_2 occurs, and that the equilibrium (1) could be established. The data at several temperatures were tested according to the equation

$$\sigma l K_1^* = \frac{A_{245}}{[\text{ClO}]^2} \quad (i)$$

where l is the absorption path length, σ the absorption cross-section and A_{245} the absorbance at 245 nm. A_{245} was found to scale with the square of the ClO concentration over ranges up to a factor of 7, independently of whether or not the mixture was being photolysed. This provides strong support for the assignment of this residual absorption to a dimeric form of ClO that is in equilibrium with ClO under our conditions.

The absorption cross-section for the dimer, σ , was determined by mass balance, assuming that all of the Cl_2O reacted was in the form ClO and Cl_2O_2 , that is, $\sigma l = 2A/(\Delta\text{Cl}_2\text{O} - \text{ClO})$. The apparent cross-section determined in this way was found to decrease with reaction time, owing to secondary reactions forming Cl_2 (mainly) and OCIO . This effect became much less noticeable at lower temperatures. By extrapolating the apparent σ values to zero time, a consistent temperature value independent of σ was obtained with an average value

$$\sigma = (6.4 \pm 0.6) \times 10^{-18} \text{ cm}^2 \text{ molecule}^{-1} \text{ at } 245 \text{ nm.}$$

This value was used to calculate the equilibrium constants K_1^* according to equation (i) from the data at different temperatures given in Table 1.

The only previous direct determination of K_1^* at 300 K gave a value of $5.1 \times 10^{-15} \text{ cm}^3 \text{ molecule}^{-1}$ (ref. 12), in good agreement

with the present data. The temperature-dependence of the equilibrium constant allows determination of the standard enthalpy and entropy changes for reaction (1): $K_p (=K^\ddagger (RT)^{-1} \text{ atm}^{-1}) = \exp(\Delta S^\ddagger / R) \exp(-\Delta H^\ddagger / RT)$. The slope and intercept of a plot of $\log_e K_p$ versus $1/T$ (Fig. 3) gave $\Delta H^\ddagger = -72.5 \pm 3.0 \text{ kJ mol}^{-1}$ and $\Delta S^\ddagger = -144 \pm 11 \text{ J mol}^{-1} \text{ K}^{-1}$.

The dimer of ClO is relatively weakly bound and at temperatures above $\sim 273 \text{ K}$, the ClO radicals that are equilibrated with Cl_2O_2 , react by the much slower bimolecular self-reactions



These reactions can account for the formation of OCIO, the dark removal of Cl_2O and formation of Cl_2 at higher temperatures, and they all have a substantial positive temperature dependence. The activation energies of the first two channels, 10a and 10b, are determined mainly by reaction endothermicities as the reverse reactions have near-zero activation energies¹³. The third channel has been investigated in our laboratory (M. Addison, J. P. Burrows, G.D.H. and R.A.C., unpublished observations), where we have now found a value of $E_{10c} = 16.1 \text{ kJ mol}^{-1}$.

These slow bimolecular channels of ClO cannot have any significant role for ClO or Cl_2O_2 loss at low temperatures. The time-dependence of Cl_2O and Cl_2O_2 at 233 K is shown in Fig. 4a. At extended photolysis times, the Cl_2O_2 absorption is seen to maximize and decline well before all the Cl_2O had been removed. Cl_2 was the final product under these conditions. The observed maximum in the Cl_2O_2 absorption can be attributed to attack of Cl atoms on Cl_2O_2



The time-dependence of $[\text{Cl}_2\text{O}]$ and $[\text{Cl}_2\text{O}_2]$ during photolysis of Cl_2 - Cl_2O_2 mixtures at 233 K can be well described by the simple mechanism involving reactions (7)+(8)+(1)+(11), although minor processes such as photolysis of Cl_2O and Cl_2O_2 affect the overall rates. The condition for the maximum in Cl_2O_2 absorption ($d[\text{Cl}_2\text{O}_2]/dt = 0$) is:

$$[\text{Cl}_2\text{O}_2]_{\text{max}} = \frac{k_8}{2k_{11}} [\text{Cl}_2\text{O}]$$

The rate constant for reaction (8) is well-established and is close to collision frequency ($k_8 = 9.8 \times 10^{-11} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ at 298 K (ref. 14). Our measurements of $[\text{Cl}_2\text{O}_2]_{\text{max}}$ and $[\text{Cl}_2\text{O}]$ give $k_8/2k_{11} = 0.54 \pm 0.01$, that is, $k_{11} = 1.06 \times 10^{-10} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$.

Figure 5 shows the full UV-absorption band that we assign to the Cl_2O_2 molecule peaking at 245 nm and extending into the near-UV region to at least 350 nm , where $\sigma \leq 1 \times 10^{-19} \text{ cm}^2 \text{ molecule}^{-1}$. Because of uncertainties arising from the scaled corrections for Cl_2 and Cl_2O , the cross-sections at the long-wavelength end ($\lambda \geq 320 \text{ nm}$) have an uncertainty of at least a factor of 2. UV spectra assigned to Cl_2O_2 have also been reported by others^{9,12}, and are shown in Fig. 5; these differ significantly from the spectrum that we attribute to Cl_2O_2 , predominantly in the Cl-O-O-Cl form. Molina and Molina⁹ used the reaction of Cl with OCIO to produce ClO in a flow system at $T \approx 240 \text{ K}$, and the spectrum was recorded under conditions chosen to give the dominant form of the ClO dimer, as judged from their interpretation of infrared spectra of the gas mixtures. Their spectrum shows similarities in some respects to the spectra obtained in the present work, when OCIO was used as a ClO precursor (see Fig. 1). But here we find that the species responsible for the absorption maximizing near 270 nm is unstable, and that a slow shift in absorption to shorter wavelengths takes place. At higher temperatures and longer reaction times, we

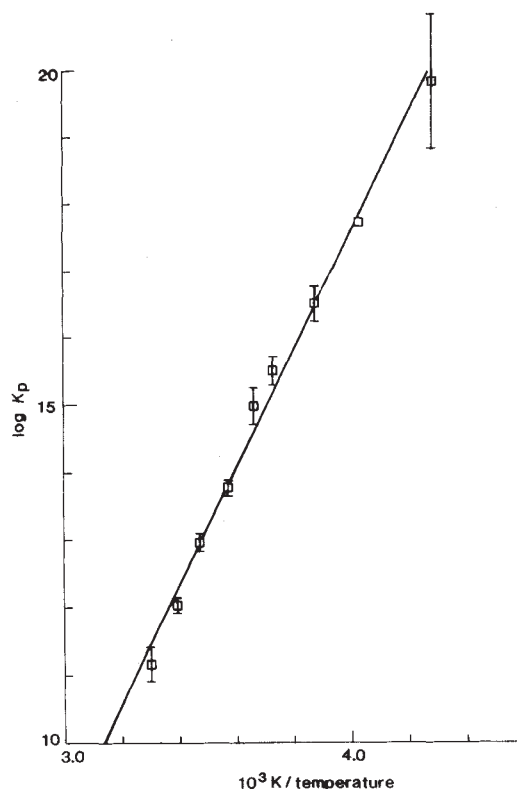


Fig. 3 A Van't Hoff plot of $\log_e K_p$, where K_p is the equilibrium constant in atm^{-1} , against the inverse of temperature for the reaction $\text{ClO} + \text{ClO} = \text{Cl}_2\text{O}_2$ in the temperature range 233 – 300 K .

have also observed spectra, using OCIO as precursor, that were similar in shape to those reported by Basco and Hunt¹² and obtained using flash photolysis of OCIO at 298 K . Further investigations of the behaviour of the OCIO photolysis system at low temperature indicate that a complex formed between OCIO and ClO contributes to the product absorption spectrum under these conditions. This work will be reported elsewhere.

Information on the photolysis products of the ClO dimer was obtained from investigation of the photolysis of Cl_2O - N_2 mixtures, using the light source providing mainly 254 nm radiation. In this case ClO radicals are produced by the reactions (8) and (9), and removal of dimer by photolysis is expected to be very much more important than when the near-UV source is used, which corresponds to much weaker absorption by the Cl_2O_2 molecule. Figure 4b shows the time-dependence of Cl_2O_2 and Cl_2O observed in this system. Unlike the Cl_2 - Cl_2O system, the rate of loss of Cl_2O accelerates with time. This cannot be attributed to increasing rate of photolysis of Cl_2O itself, because the photolytic light output was fairly constant with reaction

Table 1 Equilibrium constants for the reaction $\text{ClO} + \text{ClO} \rightarrow \text{Cl}_2\text{O}_2$

Temperature (K)	Number of observations	$K^\ddagger$ ($\text{cm}^3 \text{ molecule}^{-1} 10^{14}$)
233	5	1,310 (± 110)
248	1	169
258	15	51.1 (± 14.1)
268	17	19.6 (± 4.0)
273	15	11.9 (± 3.2)
280	3	3.67 (± 0.35)
288	15	1.68 (± 0.22)
295	3	0.67 (± 0.07)
303	3	0.29 (± 0.08)

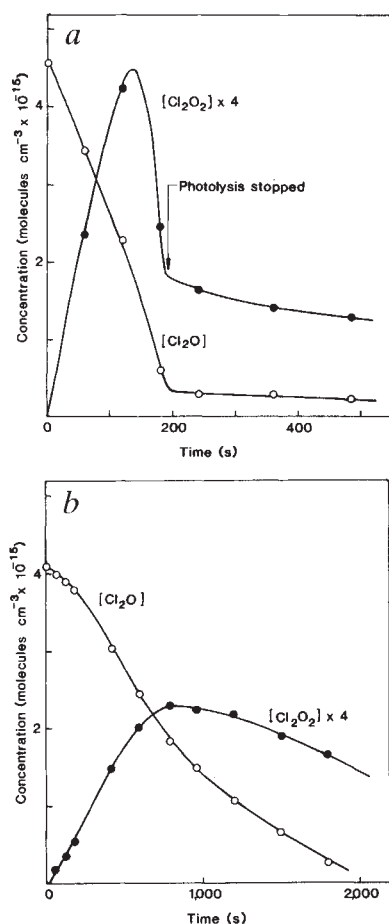
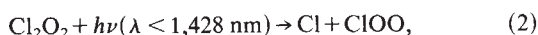


Fig. 4 *a*, Time-dependence of the Cl_2O and Cl_2O_2 concentrations when $\text{Cl}_2/\text{Cl}_2\text{O}/\text{N}_2$ mixtures are irradiated at 350 nm (temperature, 233 K). *b*, Time-dependence of the Cl_2O and Cl_2O_2 concentrations when $\text{Cl}_2\text{O}/\text{N}_2$ mixtures are irradiated at 254 nm (temperature, 233 K).

time, after an initial fall in intensity following start-up. The most likely explanation is the photodissociation of the Cl_2O_2 product to Cl atoms, which leads to further removal Cl_2O , for example



followed by reaction (3) and (8). Figure 4*b* also shows that Cl_2O_2 maximizes in a similar way to the Cl- Cl_2O system, this time due to both photolysis and Cl-atom scavenging (reaction 11). The condition for the maximum is

$$\frac{1}{2}(k_8[\text{Cl}] + k_9)[\text{Cl}_2\text{O}] = (k_{11}[\text{Cl}] + k_2)[\text{Cl}_2\text{O}_2]$$

using

$$[\text{Cl}] = (k_9[\text{Cl}_2\text{O}] + 2k_2[\text{Cl}_2\text{O}_2])/k_8[\text{Cl}_2\text{O}]$$

so we obtain

$$\frac{k_2}{k_9} = \frac{(k_{11}[\text{Cl}_2\text{O}_2]/k_8[\text{Cl}_2\text{O}] - 1)}{([\text{Cl}_2\text{O}_2]/[\text{Cl}_2\text{O}] - 2k_{11}[\text{Cl}_2\text{O}_2]^2/k_8[\text{Cl}_2\text{O}]^2)}$$

The data from three temperatures in the range 203–233 K give a value of $k_2/k_9 = 5.6(\pm 2.3)$, which is similar to the ratio of the two absorption cross-sections of Cl_2O_2 and Cl_2O at 254 nm ($\sigma_{\text{Cl}_2\text{O}_2}/\sigma_{\text{Cl}_2\text{O}} \approx 3.0$). It is concluded that the photodissociation of Cl_2O_2 at this wavelength yields Cl atoms with near-unit quantum yield, and it is probable that this applies throughout the absorption band. Small amounts of OCIO were formed in these experiments. These could not be entirely accounted for by the ClO self-reaction, channel (10*b*), and may indicate a possible minor

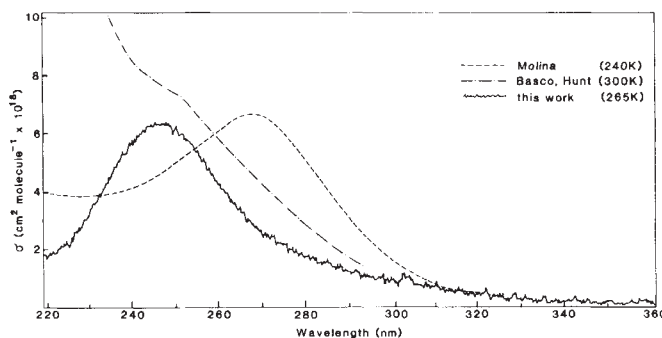


Fig. 5 UV-absorption spectrum of Cl_2O_2 in the wavelength range 220–360 nm at 265 K. The dimer absorption cross-section is given by σ . Published spectra assigned to Cl_2O_2 in ref. 9 (broken line, 240 K) and in ref. 12 (dashed dotted line, 300 K) are also shown.

contribution from the asymmetrical dimer ClOClO, either through Cl-atom scavenging or photolysis



The experimental data obtained in the present work, together with our previous measurements of k_1 (ref. 11), allow us to make some quantitative predictions concerning the behaviour of Cl_2O_2 in the Antarctic stratosphere and the efficiency of the catalytic cycle involving the ClO self-reaction. The kinetic and photochemical parameters are assumed to apply the symmetrical form of the dimer, namely ClOOCi. The rate of thermal dissociation of Cl_2O_2 by reaction (–1) can be calculated from the equilibrium constant K_1^* and the rate constant k_1 . The rate falls off rapidly with decreasing temperature, so that at the pressure and temperature (50 mbar and 200 K) corresponding to the altitude where maximum ozone depletion occurs (18–20 km), the lifetime of the Cl_2O_2 with respect to thermal dissociation is ~ 10 h.

The absorption cross-sections in the wavelength range 260–360 nm determined in this study were used to calculate the photolysis rate for Cl_2O_2 in the lower stratosphere, for specified solar zenith angles, Z , and overhead O_3 column. For $Z = 75^\circ$ and an altitude of 20 km (with O_3 120 d.u. above this height), a value of $J_2 = 1.5 \times 10^{-3} \text{ s}^{-1}$ is obtained, that is a factor of ~ 50 faster than the thermal decomposition. The temperature needs to be greater than 225 K before thermal decomposition starts to compete with photolysis, allowing equilibration between ClO and Cl_2O_2 . The rates vary quite markedly with altitude and zenith angle, and there is considerable uncertainty in J_2 arising from the uncertainty of the experimental values for σ in the long-wavelength tail of the absorption band.

It is also of interest to determine the slowest step in the overall catalytic cycle. Both reactions (3) and (4) occur on time scales of < 1 s. Because reaction (1) is second order in [ClO], its time constant is dependent on the local abundance of ClO. Both ground-based and measurements *in situ* of ClO in the Antarctic polar vortex indicate ClO concentrations of ~ 1 p.p.b. at 18–20 km altitude during the middle of September^{1,2} (that is, [ClO] is $1.8 \times 10^9 \text{ molecules cm}^{-3}$). The corresponding value of $k_1[\text{M}] \cdot [\text{ClO}]$ ($= d \log_e [\text{ClO}]/dt$) at this altitude is $4.5 \times 10^{-4} \text{ s}^{-1}$, a factor of three slower than the above value of J_2 . Thus, under these fairly typical conditions in the region of maximum ozone depletion, the results indicate that the self-reaction of ClO, reaction (1), is the slowest, and hence the rate-determining step in the catalytic cycle for ozone destruction involving the Cl_2O_2 molecule. Our results do not allow us to draw firm conclusions regarding formation of other dimeric forms of ClO with asymmetrical structures, which could provide a photolytic source of OCIO and reduce the efficiency of the catalytic cycles. The

indications are that these are minor pathways.

This work was supported by the Chemical Manufacturers Association Fluorocarbon Panel and by the UK Department of

the Environment. We thank Dr A. M. Hough for his program to calculate the atmospheric photolysis rates of Cl_2O_2 , and A. K. Lee for help with data analysis.

Received 17 December 1987; accepted 3 March 1988.

1. deZafra, R. L. *et al.* *Nature* **328**, 408–411 (1987).
2. Solomon, P. M. *et al.* *Nature* **328**, 411–413 (1987).
3. Farman, J. C., Gardiner, B. G. & Shanklin, J. D. *Nature* **315**, 207–210 (1985).
4. Stolarski, R. S. *et al.* *Nature* **322**, 808–811 (1986).
5. Callis, L. B. & Natarajan, M., *Nature* **323**, 772–777 (1986).
6. McElroy, M. B., Salawitch, R. J., Wofsy, S. C. & Logan, J. A. *Nature* **321**, 759–762 (1986).
7. Mount, G. H., Sanders, R. W., Schmeltekopf, A. L. & Solomon, S., *J. Geophys. Res.* **92**, 8320–8329 (1987).
8. Farmer, C. B., Toon, G. C., Schaper, P. W., Blavier, J.-F. & Lowes, L. L. *Nature* **329**, 126–130 (1987).
9. Molina, L. T. & Molina, M. J. *J. phys. Chem.* **91**, 433–436 (1987).
10. Barton, R. A., Cox, R. A. & Wallington, T. J. *J.C.S. Faraday Trans.* **80**, 2737–2743 (1984).
11. Hayman, G. D., Davies, J. M. & Cox, R. A. *Geophys. Res. Lett.* **13**, 1347–1350 (1986).
12. Basco, N. & Hunt, J. E. *Int. J. chem. Kinetics* **11**, 649–664 (1979).
13. Clyne, M. A. A. & Watson, R. T. *J.C.S. Faraday Trans.* **75**, 1169–1187 (1977).
14. DeMore, W. B. *et al.* *Chemical Kinetics and Photochemical Data for use in Stratospheric Modelling* (Jet Propulsion Laboratory, California, Publication 85–37, 1985).

A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides

Raymond J. Deshaies*, Bruce D. Koch*, Margaret Werner-Washburne†, Elizabeth A. Craig† & Randy Schekman*

* Department of Biochemistry, University of California, Berkeley, California 94720, USA

† Department of Physiological Chemistry, School of Medicine, University of Wisconsin, Madison, Wisconsin 53706, USA

Depletion of a subset of 70K stress proteins in yeast mutants shows that they are involved in the post-translational import of precursor polypeptides into both mitochondria and the lumen of the endoplasmic reticulum. The identification of such a basic function may explain the remarkable evolutionary conservation of the gene family encoding these proteins.

SYNTHESIS of heat shock proteins (hsp) is a conserved response to environmental stress that is found in all organisms (reviewed in refs 1 and 2). Among the proteins whose synthesis is stress-induced, the family of polypeptides of relative molecular mass 70,000 (M_r 70K, hsp70s) has been best studied. Genes encoding these proteins were first isolated from *Drosophila*, and have since been found in every organism tested, including *Escherichia coli*³, yeast⁴, and humans⁵. Eukaryotic organisms typically contain several hsp70-related sequences. For example, the *Drosophila* genome harbours eight homologous genes (K. Palter and E.A.C., unpublished results), the human genome potentially encodes ten genes⁵, and the simple eukaryote *Saccharomyces cerevisiae* contains at least eight genes⁶. Regulation of these genes is complex; some hsp70 genes are expressed only after cells are stressed, whereas others are expressed constitutively. Though the function of hsp70s remains obscure, it is assumed that they perform important activities due to the extraordinary conservation of their primary structure and expression among diverse organisms. Indeed, genetic analysis of the hsp70 gene family in yeast indicates that two subfamilies are essential for vegetative growth^{6,7}.

A variety of functions have been postulated for 70K stress proteins based on their biochemical characteristics. A common property of hsp70 proteins is their ability to bind ATP with high affinity^{8,9}. A member of the 70K stress protein family catalyses an ATP-dependent depolymerization of clathrin from coated vesicles *in vitro*^{8,10,11}. ATP hydrolysis has also been implicated in the functions of the major heat-inducible hsp70, which is released from tight binding sites in the nucleolus by micromolar concentrations of hydrolysable ATP¹². Lastly, ATP disrupts the association between the hsp70-like immunoglobulin heavy chain binding protein¹³ and immunoglobulin heavy chains in IgG immunoprecipitates¹⁴. These properties of the 70K stress proteins led Pelham to suggest that hsp70s couple ATP hydrolysis to the disruption of quaternary structure in multi-subunit com-

plexes or denatured aggregates¹⁵. A simple extension of this model could accommodate a function for hsp70s in the relaxation of tertiary structure within a single polypeptide chain.

Post-translational import of precursor proteins into mitochondria requires both NTP hydrolysis outside the mitochondrial inner membrane^{16–18} and 'unfolding' of the transported polypeptide¹⁹. Rothman and Kornberg suggested that the energy of phosphodiester bond cleavage may be harnessed by a factor that unravels mitochondrial precursors to ensure a translocation-competent conformation²⁰. We speculated that an hsp70-like protein might function as this 'unfoldase' and that hsp70s might also act in the post-translational import of certain secretory proteins into the lumen of the endoplasmic reticulum (ER^{21–26}). To test this hypothesis, we examined ER and mitochondrial biogenesis in *S. cerevisiae* strains bearing mutated hsp70 genes. Here we show that yeast cells that were depleted of a subset of hsp70 polypeptides accumulated precursor forms of proteins normally destined for import into the ER and mitochondria. Proteins extracted from these depleted cells failed to sustain import into the yeast ER *in vitro*. In an independent effort, W. Chirico, G. Waters, and G. Blobel in the accompanying article show that highly purified hsp70 proteins participate in secretory protein translocation⁴⁹. These results indicate that hsp70s function in post-translational import pathways, and suggest that the early stages of protein import into two distinct organelles are mechanistically similar.

Consequences of Hsp70 depletion *in vivo*

DNA hybridization studies have revealed eight hsp70-related genes in *S. cerevisiae*⁴. These genes are all transcribed⁶, and their sequences predict polypeptides of relative molecular masses ~70K. Based on sequence comparisons and the phenotypes of strains containing hsp70 gene disruptions, these genes are divided into four groups denoted SSA–SSD⁶. Our

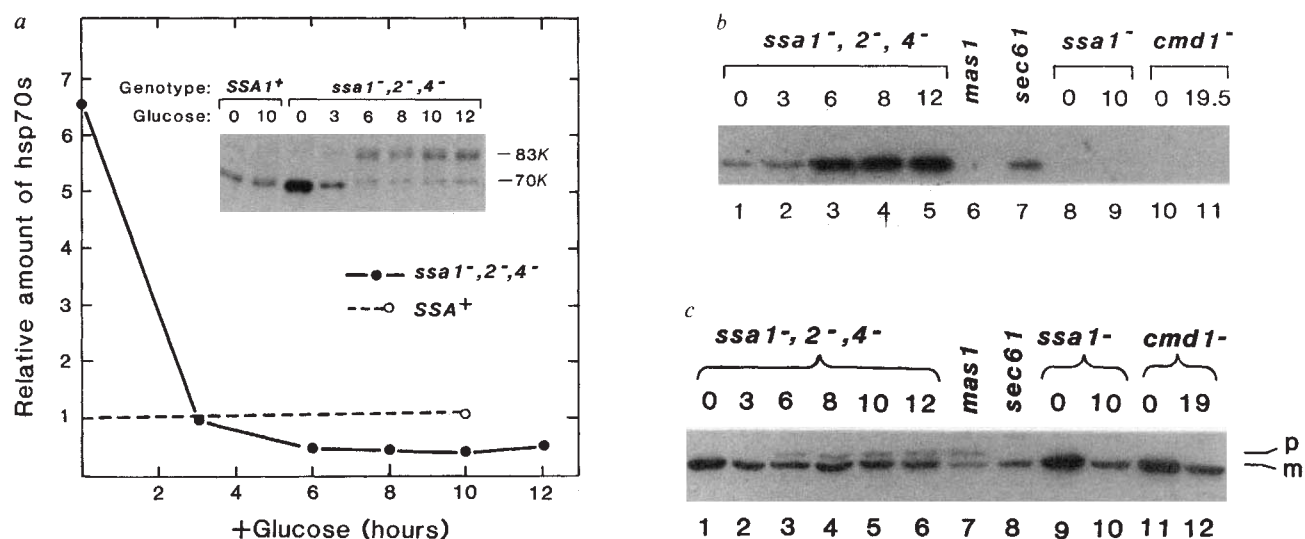


Fig. 1 Depletion of yeast hsp70s results in the accumulation of precursor proteins normally destined for the endoplasmic reticulum and mitochondria. **a**, Hsp70 antigen present in DS10 ($SSA1^+$ (○); inset, lanes 1 and 2) and MW141 ($ssa1^{-}, 2^{-}, 4^{-}$ (●); inset, lanes 3–8) cells was measured by immunoblotting extracts prepared 0, 3, 6, 8, 10 and 12 h after supplementing each culture with 3% glucose. An autoradiogram of the quantified immunoblot is depicted in the inset. 70K, yeast hsp70s; 83K, yeast hsp83 and hsc83. **b**, MW141 samples from each time point depicted in **a** were analysed for their content of the secretory precursor protein prepro- α -factor by immunoblotting. Cells bearing mutant copies of the *mas1*, *sec61*, and *ssa1* genes, as well as a strain (*cmd1*[−]) that ceased growth after ~19 h in glucose medium due to repression of calmodulin synthesis, were also analysed. The numbers above each lane indicate hours elapsed after the addition of glucose. **c**, Same as **b**, except the nitrocellulose filters were probed with antiserum that recognized the β -subunit of the mitochondrial F_1 ATPase. p, $F_1\beta$ precursor; m, mature $F_1\beta$.

Methods. *S. cerevisiae* strains MW141 (*ura3-52, trp1, leu2-3,112, his3-11,15, ssa1::HIS3, ssa2::LEU2, ssa4::URA3, MAT α*) transformed with *pGAL1-SSA1*, which contained the *SSA1* structural gene fused 3' to the *GAL1* promoter⁶, MW146 (*ura3-52, trp1, leu2-3,112, his3-11,15, ssa1::HIS3, MAT α*)⁶, DS10 (*ura3-52, trp1, leu2-3, 112, his3-11, 15, lys2, MAT α*) and TDY21-6A (*leu2-3, 112, his, lys2, trp1, ura3-52, cmd1::TRP1, MAT α* , transformed with pTD39, which contained the *Xenopus* calmodulin structural gene fused 3' to the yeast *GAL1* promoter; T. Davis and J. Thorner, personal communication) were grown in YPG (1% Bacto-yeast extract, 2% Bacto-peptone and 2% galactose) at 30 °C to early logarithmic phase, supplemented with glucose to a final concentration of 3% and incubated at 30–32 °C. At various times after the addition of glucose, samples (8×10^7 cells) were removed, adjusted to 10mM Na₂S₂O₃ and placed on ice. Whole cell extracts were prepared by glass-bead lysis as described³³. Protein was measured by the Lowry method⁴⁵, and an equal portion from each extract (40 μ g) was loaded on a 7.5% (**a** and **c**) or 12.5% (**b**) SDS-polyacrylamide gel, electrophoresed⁴⁶, transferred to nitrocellulose⁴⁷, and immunoblotted³⁰ with antisera directed against: **a**, C-terminal portion of Ssa1p and Ssa2p (M. Werner-Washburne, unpublished data); **b**, prepro- α -factor²³; and **c**, β subunit of F_1 ATPase⁴⁸. Filter-bound antibodies were probed with ¹²⁵I-protein A⁴⁷, and decorated proteins were visualized by autoradiography and quantified as described³⁰. *S. cerevisiae* strains 15-5B (*leu2-3,112, ade2, ura3-52, pep4-3, sec61, MAT α*)³⁰ and RSY129 (*mas1-1, leu2-3,112, his3, MAT α* ; M. Yaffe, personal communication) were grown in YPD (same as YPG, except glucose is substituted for galactose) at 24 °C to early logarithmic phase. Both cultures were shifted to 37 °C for 2 h, and extracts were prepared and evaluated as described above.

discussion focuses on the *SSA* subgroup, which contains four members, *SSA1*–*SSA4*. The genetic interactions between these genes suggest that the *SSA* gene products perform interchangeable functions *in vivo*⁶. *SSA1* and *SSA2* are transcribed constitutively, though the level of *SSA1* messenger RNA increases several-fold after a heat shock. *SSA3* and *SSA4* are only expressed in cells exposed to high temperatures⁶. Whereas yeast strains bearing single disruptions in any one of the four genes are phenotypically indistinguishable from wild type, *ssa1 ssa2* strains grow slowly at 24 °C and do not grow at 37 °C (ref. 27), and *ssa1 ssa2 ssa4* cells are inviable. Thus, the *SSA* genes define a subgroup of hsp70-related loci in *S. cerevisiae* that perform an essential function⁶.

To examine the consequences of an *SSA* deficiency in more detail, a strain that conditionally expresses a single *SSA* gene was generated. This strain, MW141, harbours chromosomal disruptions of the *SSA1*, *SSA2* and *SSA4* genes, but is rescued by a single copy of *SSA1* present on a centromeric plasmid. This plasmid contains the coding region of *SSA1* fused to the yeast *GAL1* promoter⁶. MW141 cells grown in galactose overproduced *SSA1* protein (Ssa1p) ~sixfold (Fig. 1a). When these cells were shifted to medium containing glucose, the *GAL1* promoter was repressed²⁸, and the steady state level of Ssa1p declined rapidly (Fig. 1a). Three hours after the addition of glucose, the amount of Ssa1p detected on immunoblots was

reduced to wild type levels; by 6–8 h, the depletion was nearly complete. The residual immunoreactivity may have been due to other hsp70-like proteins, since the anti-Ssa1p serum cross-reacted with several yeast hsp70 homologues (M. Werner-Washburne, unpublished results).

To address whether this depletion of Ssa1p interfered with the biogenesis of secretory and mitochondrial proteins, extracts prepared from cells at various stages of Ssa1p depletion were immunoblotted with antisera directed against the secretory protein prepro- α -factor (Fig. 1b) and the β -subunit of the mitochondrial protein F_1 ATPase ($F_1\beta$; Fig. 1c). In wild type cells, α -factor precursor is rapidly translocated, core-glycosylated, proteolytically processed, and secreted²⁹. Prepro- α -factor did not accumulate in wild type cells (data not shown, see ref. 30) or in strains lacking only the *SSA1* gene (*ssa1*[−], Fig. 1b, lanes 8 and 9). MW141 cells grown in galactose accumulated a low steady-state level of prepro- α -factor (Fig. 1b, lane 1). After Ssa1p dropped below wild type levels, there was a dramatic increase in prepro- α -factor accumulation (Fig. 1b, lanes 3–5). Precursor accumulated in MW141 cells was indistinguishable from that detected in the translocation-defective mutant *sec61*³⁰ (Fig. 1b, lane 7). Prepro- α -factor accumulation was not merely the result of cell death following a shift to glucose medium, since strains that had ceased growth due to glucose-dependent depletion of calmodulin (Fig. 1b, lanes 10 and 11), or the ER

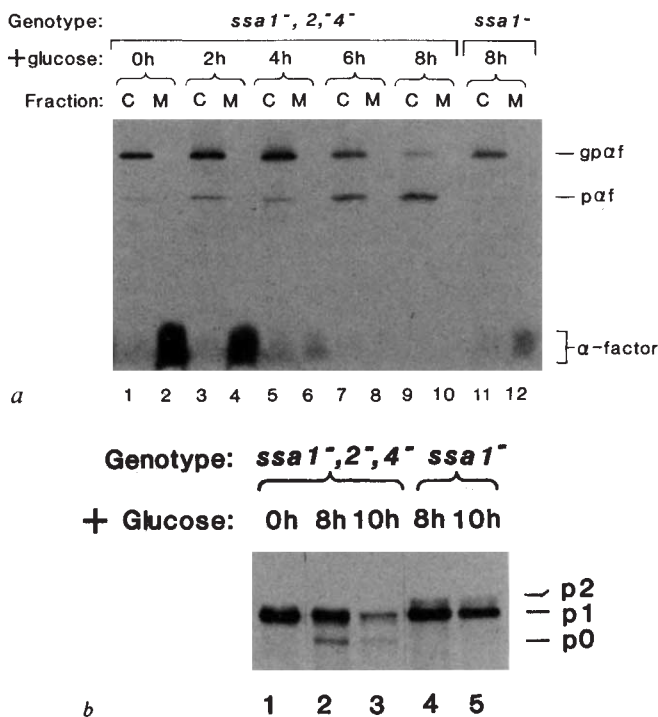


Fig. 2 Precursor forms of secretory and vacuolar proteins accumulate in yeast cells depleted of hsp70s. *a*, Cells progressively depleted of hsp70s (*ssa1-2-4-*, lanes 1–10) and control cells (*ssa1-*, lanes 11 and 12) were radiolabelled with [^{35}S]SO $_4^{2-}$ and the cultures were separated into cell pellet (odd-numbered lanes) and extracellular supernatant (even-numbered lanes) fractions. Each fraction was assayed for its content of prepro- α -factor by immunoprecipitation and SDS-PAGE. The position of unmodified prepro- α -factor, paf, is indicated; gpaf refers to core-glycosylated pro- α -factor in transit to the cell surface, and α -factor refers to the mature, processed pheromone. *b*, Radiolabelled cell pellet fractions prepared in parallel with those shown in *a* were immunoprecipitated with anti-carboxypeptidase Y serum³⁰ and the immune complexes were evaluated by SDS-PAGE. Lanes 1–3, MW141 (*ssa1-2-4-*) cells labelled 0, 8 and 10 h after the addition of glucose. Lanes 4 and 5, MW146 (*ssa1-*) cells labelled 8 and 10 h after the addition of glucose. p1, core-glycosylated procarboxypeptidase Y in transit through the endoplasmic reticulum; p2, procarboxypeptidase Y that has received additional glycosyl modifications in the Golgi body; p0, unmodified preprocarboxypeptidase Y.

Methods. MW141 and MW146 cells (see Fig. 1 Methods) were grown in selective minimal medium plus 2% galactose at 30 °C to early logarithmic phase. Glucose was added to each culture at a final concentration of 3%, and, at the indicated times, 3×10^7 cells from each culture were harvested and labelled with [^{35}S]SO $_4^{2-}$ for 6 min (*b*) or 15 min (*a*). The radiolabelling was terminated by the addition of an equal volume of ice cold 20 mM NaN $_3$, and the quenched reactions were separated into cell pellet and culture medium fractions. The culture medium from the 15 min pulse was adjusted to 0.4% SDS, heated, and immunoprecipitated with anti- α -factor serum as described³⁰. Both pellet fractions were simultaneously processed by glass-bead lysis and immunoprecipitation³⁰ with anti- α -factor (*a*) or anti-carboxypeptidase Y sera (*b*), and immune complexes were evaluated by SDS-PAGE⁴⁶ on 12.5% (*a*) or 7.5% (*b*) gels. Following electrophoresis, gels were treated with Amplify (Amersham) dried, and autoradiographed at –70 °C.

protein HMG-CoA reductase (M. Basson and J. Rine, personal communication) showed no such defect.

F $_1\beta$ is synthesized as a precursor containing a 19 amino acid presequence that is cleaved upon import into mitochondria³¹. MW141 cells grown in galactose did not accumulate F $_1\beta$ precursor (Fig. 1c, lane 1), but as Ssa1p fell below wild type levels, preF $_1\beta$ was detected (lanes 3–6). PreF $_1\beta$ accumulated in MW141 cells comigrated with F $_1\beta$ precursor detected in the

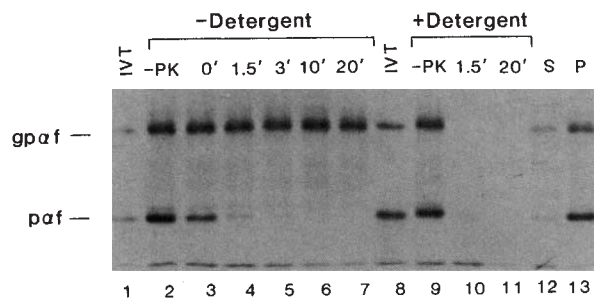


Fig. 3 Prepro- α -factor accumulated in hsp70-depleted cells is exposed to the cytoplasmic compartment. MW141 (see Fig. 1, Methods) cells shifted to glucose for 6 h were radiolabelled with [^{35}S]SO $_4^{2-}$ and an extract was prepared by gentle lysis of spheroplasts. The extract was either mock-digested (lanes 2 and 9) or exposed to 300 $\mu\text{g ml}^{-1}$ proteinase K at 4 °C for 0–20 min in the absence (lanes 3–7) or presence (lanes 10 and 11) of 0.4% Triton X-100. To assess the integrity of the endoplasmic reticulum membrane, aliquots of cell-free extract were separated into supernatant (lane 12) and pellet (lane 13) fractions. As a migration standard, samples derived by *in vitro* translation of prepro- α -factor mRNA in the presence of yeast microsomes^{21–23} were included (lanes 1 and 8).

Methods. MW141 cells were grown in selective minimal medium plus 2% galactose at 30 °C to early logarithmic phase. Glucose was added to a final concentration of 3%, and after 6 h, 13×10^7 cells were harvested and radiolabelled with [^{35}S]SO $_4^{2-}$ for 30 min at 30 °C. Radioactive incorporation was terminated by the addition of NaN $_3$ to 10 mM, and spheroplasts were prepared, lysed by homogenization, and extracts clarified by centrifugation exactly as described³⁰. Aliquots (1×10^7 cell-equivalents per time point) of the cell-free supernatant were subjected to proteolysis as detailed above. An untreated sample was fractionated by centrifugation for 10 min at 12,000g. The soluble and particulate fractions were processed as described below. Proteinase treatments were terminated by the addition of trichloroacetic acid to a final concentration of 20%, and precipitated proteins were washed, solubilized, and subjected to immunoprecipitation with anti- α -factor serum as described³⁰. Immune complexes were analysed by SDS-PAGE and fluorography as described in the Fig. 2 legend.

mas1 mutant, which is defective in cleaving presequences of proteins targeted to mitochondria³². Neither wild type (data not shown) nor *ssa1 SSA2 SSA4* strains (Fig. 1c, lanes 9 and 10) accumulated detectable amounts of preF $_1\beta$. Similarly, cells that ceased growth upon shift to glucose medium due to depletion of calmodulin (Fig. 1c, lanes 11 and 12) did not contain preF $_1\beta$. The mutants^{30,33} *sec61* (Fig. 1b, lane 7 and Fig. 1c, lane 8), *mas1* (Fig. 1b, lane 6; Fig. 1c, lane 7), *sec62* and *mas2* (data not shown) exhibit organelle-specific defects in protein import. In contrast, reduction of Ssa1p simultaneously blocks correct processing of precursors targeted to both the ER and mitochondria.

The experiments described above reveal steady state precursor accumulation in cells depleted of Ssa1p. To evaluate directly the biogenesis of different secretory precursor proteins, a more sensitive assay was employed. MW141 cells were pulse-labelled with [^{35}S]SO $_4^{2-}$ at various times after the repression of *SSA1* transcription, and radiolabelled extracts were immunoprecipitated with sera directed against α -factor (Fig. 2a) and the vacuolar protein carboxypeptidase Y (Fig. 2b). MW141 cells grown in galactose medium accumulated a small fraction of newly synthesized α -factor as prepro- α -factor (Fig. 2a, lane 1), though the majority of the pheromone precursor was efficiently processed and secreted (lane 2). After increasing periods of time in glucose medium, a larger proportion of newly synthesized α -factor was trapped intracellularly as the unprocessed prepro-pheromone (Fig. 2a, lanes 3–10). In contrast, *ssa1 SSA2 SSA4* cells shifted to glucose for 8 h did not produce detectable levels of unprocessed prepro- α -factor (Fig. 2a, lane 11). Evaluation of carboxypeptidase Y biogenesis in MW141 cells progressively

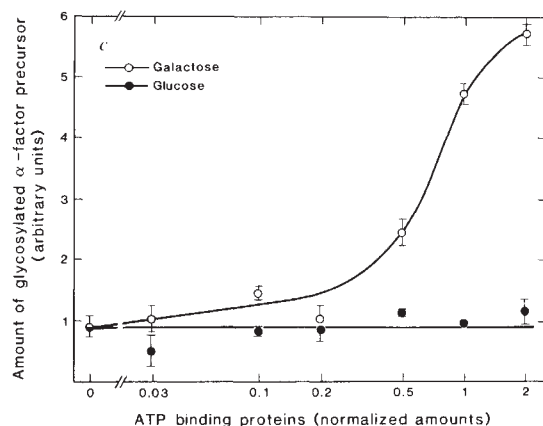
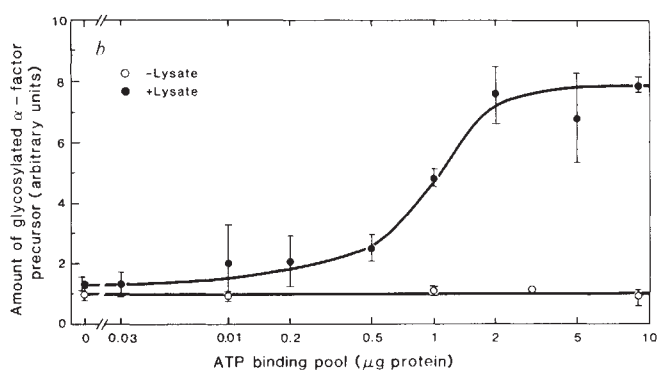
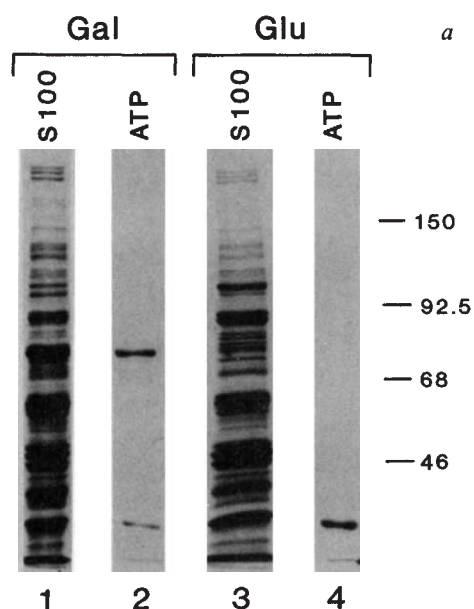


Fig. 4 ATP-binding proteins and an additional cytosolic factor(s) stimulate protein translocation into yeast microsomes. *a*, ATP-binding proteins (lanes 2 and 4) were prepared from the cytosol (lanes 1 and 3) of MW141 cells that contained (lanes 1 and 2) or were depleted (lanes 3 and 4) of Ssa1p. The crude cytosol, (75 μg per lane) and enriched protein fractions (lane 2, 3.4 μg; lane 4, 2 μg) were subjected to SDS-PAGE on 7.5% gels, and proteins were visualized by staining with Coomassie Blue. *b*, The effect of ATP-binding proteins from MW141 cells containing Ssa1p upon translocation into the ER was examined in the absence (○) and presence (●) of 21 μg postribosomal supernatant protein extracted from MW141 cells. The amount of glycosylated pro- α -factor produced in an *in vitro* post-translational translocation reaction was used to measure import into the ER. Each point represents the mean $\pm$ s.e.m. ($n = 2$). *c*, The ability of ATP-binding proteins isolated from either Ssa1p-containing (○) or Ssa1p-depleted (●) MW141 cells to stimulate post-translational translocation was examined in the presence of 12 μg postribosomal supernatant protein prepared from MW141 cells. Each point represents the mean $\pm$ s.e.m. ($n = 2$).

Methods. ATP-binding proteins were prepared from MW141 cells (see Fig. 1, Methods) grown at 25 °C (*b*) or 30 °C (*a* and *c*) by ATP-agarose chromatography⁸. Where indicated, Ssa1p was depleted *in vivo* by adding 30 g glucose per litre of culture 10.5 h before harvesting. To assess recovery in *c*, each culture was supplemented with a trace amount of [³⁵S]-labelled galactose-grown MW141 cells before the lysis step. Cytosol protein (206 mg) extracted from each culture was loaded onto 2 ml ATP-agarose columns in parallel. The amount of the resulting radioactive ATP-binding proteins added to each reaction shown in *c* was normalized as a fraction of the total radioactivity in the crude cytosol. The maximal amount of ATP-binding proteins assayed in *c* was 6 μg. The postribosomal supernatant fraction was prepared from MW141 grown at 30 °C to which 30 g glucose per litre of culture had been added 10 h previously. Cells were lysed in a Bead-Beater (Biospec Products), and the homogenate was centrifuged and gel filtered²¹. Protein concentrations were determined with the Biorad protein assay. Microsomal membranes were prepared²³ from wild type yeast strain RDM15-9B³⁰. mRNA encoding prepro- α -factor²² was translated in a wheat germ lysate (Amersham) and post-translational translocation assayed with the indicated additions and 50 μM GDP-mannose³⁴. Translocation reactions were run on a 12.5% SDS-polyacrylamide gel. The gels were fixed, soaked in Amplify (Amersham), and exposed to film. The sections of the resulting autoradiograms corresponding to pro- α -factor with three core oligosaccharides were quantified by densitometric scanning³⁰.

depleted of Ssa1p revealed a precursor form (pO; Fig. 2*b*, lanes 2 and 3) that was not detected in *ssa1 SSA2 SSA4* cells. This precursor comigrated with unmodified preprocarboxypeptidase Y synthesized in the translocation-defective mutants *sec61* and *sec62* (data not shown). MW141 cells also contained a precursor form of the secretory protein invertase that was not detected in wild type strains (data not shown), although as with preprocarboxypeptidase Y, the dependence on Ssa1p was not as dramatic as for prepro- α -factor maturation.

Ssa1p depletion could have blocked either translocation of prepro- α -factor into the ER, or its subsequent covalent modification within the lumen of this organelle. To distinguish between these possibilities, the accessibility of newly synthesized prepro- α -factor to exogenously added protease was examined. MW141 cells exposed to glucose for 6 hours were radiolabelled with [³⁵S]SO₄, and a lysate was prepared by homogenization of spheroplasts. To assess the disposition of the accumulated precursors, the lysate was exposed to proteinase K either in the presence or absence of detergent. Glycosylated pro- α -factor

sequestered within the lumen of the ER was refractory to digestion, whereas prepro- α -factor accumulated in response to hsp70 deprivation was degraded completely (Fig. 3, lanes 2–7). The glycosylated pro- α -factor was not intrinsically resistant to protease, since solubilization of the ER membrane with detergent abolished its protection (Fig. 3, lanes 9–11). Cytoplasmically exposed prepro- α -factor sedimented with the particulate fraction (Fig. 3, lane 13). This sedimentation may have resulted from a specific association with the ER membrane, or non-specific aggregation of accumulated precursors. We failed to document that accumulated F₁ β precursor was exposed to the cytosol, since this precursor was inherently labile in spheroplast homogenates prepared from MW141 cells.

Ssa1p and translocation *in vitro*

α -Factor precursor made in a wheat germ lysate is assembled into yeast ER vesicles poorly, but uptake is stimulated approximately fivefold by a soluble extract from yeast³⁴. To test whether Ssa1p was required for maximal ER import *in vitro*, we partially

purified Ssa1p and assayed its ability to stimulate translocation of wheat germ-derived prepro- α -factor into yeast microsomes.

Soluble proteins were extracted from MW141 cells grown in galactose. Overproduced Ssa1p was readily identifiable in these extracts as a species of ~70K (Fig. 4a, lane 1). ATP-affinity chromatography of this lysate highly enriched the Ssa1p (Fig. 4a, lane 2). This partially purified fraction failed to stimulate translocation of wheat germ-derived prepro- α -factor into yeast microsomes (Fig. 4b, open circles). If the ATP-binding proteins were assayed in the presence of a low level of a yeast postribosomal supernatant fraction, however, a synergistic stimulation of prepro- α -factor import into yeast ER vesicles was observed (Fig. 4b, closed circles). Therefore, post-translational transfer of prepro- α -factor across the ER membrane *in vitro* required both an ATP-binding protein and another factor(s).

To determine whether Ssa1p depletion reduced the translocation-stimulating activity of the ATP-binding protein fraction, soluble proteins were extracted in parallel from MW141 cells either grown in galactose medium or shifted to glucose medium for ten hours. SDS-PAGE analysis of the unfractionated lysates revealed a marked glucose-dependent depletion of a ~70K protein (Fig. 4a, lanes 1 and 3). ATP affinity chromatography of these lysates yielded the enriched ATP-binding protein fractions shown in Fig. 4a, lanes 2 and 4. Normalized amounts of these ATP-binding proteins were assayed in the heterologous prepro- α -factor translocation reaction in the presence of a low level of the yeast postribosomal supernatant fraction (Fig. 4c). Near maximal stimulation was obtained with proteins isolated from MW141 cells grown in galactose (open circles), whereas the Ssa1p-deficient fraction failed to enhance import into yeast ER vesicles. Independent purification of the translocation-stimulating activity of the ATP-binding protein fraction has yielded a homogeneous preparation of Ssa1p and Ssa2p⁴⁹ (Chirico *et al.*, accompanying article⁴⁹). Our observation that ATP-binding proteins extracted from galactose-grown MW141 cells support ER import *in vitro* (Fig. 4b, c), show that Ssa1p by itself fulfils the requirement for hsp70s.

Conclusions

Yeast cells depleted of a subset of hsp70-like polypeptides (Ssa1p, Ssa2p, and Ssa4p) contain unprocessed precursors of proteins destined for mitochondria (F₁F₀) or the ER (prepro- α -factor, preprocarboxypeptidase Y). Accumulated prepro- α -factor is exposed to the cytosol, indicating that depletion of SSA gene products interrupts secretion during protein translocation into the ER. Ssa2p and Ssa4p appear to overlap the function of Ssa1p *in vivo* inasmuch as *ssa1 ssa2 ssa4* (Fig. 1b, lanes 8 and 9; Fig. 1c, lanes 9 and 10) and *ssa1 ssa2 ssa4* (data not shown) strains accumulate neither prepro- α -factor nor preF₁F₀. SSA proteins may affect organelle biogenesis indirectly by influencing the production of another active factor required for protein translocation. However, purified yeast hsp70-like proteins stimulate translocation of prepro- α -factor into yeast microsomes *in vitro*, supporting a direct role for SSA proteins in ER and, by inference, in mitochondrial protein import.

Members of the hsp70 family may be responsible for the recovery of nucleolar morphology following heat stress³⁵ and the removal of clathrin triskelions from endocytic coated vesicles^{8,11}. Given the large number of hsp-related sequences in the yeast⁴, *Drosophila*², human⁵ and mouse³⁶ genomes, it seems reasonable to propose that hsp70s execute a diverse array of cellular functions. The high degree of sequence homology detected among yeast or *Drosophila* hsp70 genes implies that some hsp70 polypeptides share overlapping activities or perform similar activities in different compartments. SSA gene products, for example, appear to function both in mitochondrial and ER import pathways, though each may have distinct primary responsibilities while retaining the capacity to act interchangeably.

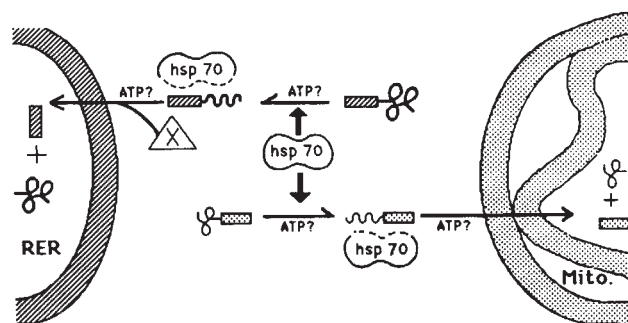


Fig. 5 Proposed role of hsp70s in protein translocation. One or more hsp70 binds to a secretory or mitochondrial precursor polypeptide. The position of this interaction is unknown, as indicated by the dotted lines at the hsp70-precursor interface. Binding may occur as the precursor is being made or after completion of the polypeptide. The initial interaction may consume ATP, or ATP may be bound but not hydrolysed by the complex. For import into the ER, at least one additional yeast lysate component is required (factor X). We propose that this factor(s) targets secretory proteins to the ER membrane. A translocation-competent complex then associates via a signal or transit peptide with the proper target membrane and as penetration begins the complex dissociates allowing the hsp70 to be recycled. ATP hydrolysis may facilitate this dissociation step.

Dissection of mitochondrial and ER post-translational protein import reactions has revealed that both processes require phosphodiester bond cleavage in the cytosol^{16-18,21-25}. In addition, translocation of precursors into mitochondria requires that the transported protein retain an import-competent structure; stabilization of the tertiary structure of a heterologous leader-bearing fusion protein specifically interferes with its import into mitochondria¹⁹. Two lines of evidence suggest that these requirements are related by the use of ATP to power an obligatory unfolding reaction during protein translocation. First, nascent chains (presumably unfolded) have a relaxed requirement for ATP in an *in vitro* mitochondrial import reaction³⁷. Second, M13 phage coat protein³⁸ and the mitochondrial inner membrane ADP/ATP carrier¹⁶ translated in a reticulocyte lysate show enhanced sensitivity to protease digestion in the presence of NTPs. We propose that this unfolding of precursor proteins prior to their import into mitochondria and the ER is catalysed by a member of the 70K stress protein family.

Translocation of secretory polypeptides into ER vesicles derived from mammalian cells requires both signal recognition particle (SRP) and its receptor located on the cytoplasmic face of the ER membrane (reviewed in ref. 39). While no requirement for an hsp70-like protein has been identified, it is possible that SRP possesses an intrinsic capacity to inhibit folding of nascent chains. Alternatively, cotranslational assembly pathways may have a relaxed dependence on 'unfoldases'. Perhaps hsp70 function is required only by proteins that are imported post-translationally. In this regard, prepro- α -factor may be imported mainly after translation, whereas preprocarboxypeptidase Y and preinvertase, which are less dramatically affected by hsp70 depletion, may be translocated co-translationally. These speculations are supported by data from *in vitro* protein translocation studies: prepro- α -factor is efficiently imported into yeast microsomes post-translationally, whereas preinvertase and preprocarboxypeptidase Y are only translocated efficiently in a co-translational reaction⁴⁰. Participation of hsp70-like proteins in polypeptide translocation into mammalian ER may be explored with ribosome-tethered nascent chains that can be imported post-translationally^{24,25}.

Soluble factors that stimulate translocation of precursors across target membranes have been reported in several other systems. One of these, trigger factor, forms a complex with renaturing pro-OmpA to facilitate translocation into *E. coli* cytoplasmic membrane vesicles⁴¹. This protein may correspond

to the activity that suppresses folding of presursors that accumulate within mutant *E. coli* cells⁴². Weich *et al.* have identified an activity present in reticulocyte lysate that is required for the ATP-dependent, SRP-independent post-translational insertion of M13 procoat into dog pancreas microsomes³⁸. Finally, the mitochondrial ATPase β subunit precursor either purified from cells⁴³ or made *in vitro*⁴⁴ is imported optimally in the presence of a soluble factor from yeast or rabbit reticulocyte lysates. This requirement does not seem to be universal, however, for hybrid proteins made in and purified from *E. coli* are able to enter mitochondria in the absence of cytosol¹⁹. Since urea denaturation of such hybrid proteins dramatically stimulates the rate of their assembly into mitochondria (M. Eilers and G. Schatz, personal communication), a similar enhancement of translocation may be produced by 70K stress proteins.

The simultaneous requirement for hsp70 function in the proper localization of proteins to distinct subcellular compartments suggests that hsp70s fulfil a general mechanistic requirement for the transfer of precursor proteins across target membranes. A model that extends upon previous work and incorporates these new proposals is presented in Fig. 5. This hypothesis

can easily be accommodated within a model for hsp70 function suggested by Pelham¹⁵. The ATP-driven cycle of hsp70 binding to and release from substrates targeted to the mitochondria or ER may act to dissolve aggregates/multimers formed by untranslocated precursors⁴⁴, or to relax the tertiary structure of polypeptides prior to membrane penetration. Alternatively, hsp70 may contact unfolded nascent chains and prevent them from adopting conformations incompatible with translocation. Lastly, hsp70 action may result in a specific precursor configuration required for membrane assembly. The interaction of hsp70s with precursor proteins and the conformation that precursors adopt under the influence of hsp70s can now be examined with purified components.

We thank David Stone, Michael Basson, Tricia Davis and Michael Yaffe for strains, Michael Douglas and Michael Yaffe for anti-F₁ β serum, Bill Hansen for pDJ100, Peggy McCutchan-Smith for help in preparing this manuscript, and members of the Schekman lab for critically reading this paper. This work has been supported by a grant from the Jane Coffin Childs Memorial Fund for Medical Research to B.K. and NIH grants GM26755 and GM36881 to R.S.

Received 2 February; accepted 9 March 1988.

1. Lindquist, S. *A. Rev. Biochem.* **55**, 1151-1191 (1986).
2. Craig, E. A. *Crit. Rev. Biochem.* **18**, 239-280 (1985).
3. Bardwell, J. C. A. & Craig, E. A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 848-852 (1984).
4. Ingolia, T. D., Slater, M. R. & Craig, E. A. *Molec. cell. Biol.* **2**, 1388-1398 (1982).
5. Mues, G. I., Munn, T. Z. & Raese, J. D. *J. biol. Chem.* **261**, 874-877 (1986).
6. Werner-Washburne, M., Stone, D. E. & Craig, E. A. *Molec. cell. Biol.* **7**, 2568-2577 (1987).
7. Craig, E. A., Kramer, J. & Kosic-Smithers, J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4156-4160 (1987).
8. Chappell, T. G., Welch, W. J., Schlossman, D. M., Palter, K. B., Schlesinger, M. J. & Rothman, J. E. *Cell* **45**, 3-13 (1986).
9. Welch, W. J. & Feramisco, J. R. *Molec. cell. Biol.* **5**, 1229-1237 (1985).
10. Schlossman, D. M., Schmid, S. L., Braell, W. A. & Rothman, J. E. *J. Cell Biol.* **99**, 723-733 (1984).
11. Ungewickell, E. *EMBO J.* **4**, 3385-3391 (1985).
12. Lewis, M. J. & Pelham, H. R. B. *EMBO J.* **4**, 3137-3143 (1985).
13. Bole, D. G., Hendershot, L. M. & Kearney, J. F. *J. Cell Biol.* **102**, 1558-1566 (1986).
14. Munro, S. & Pelham, H. R. B. *Cell* **46**, 291-300 (1986).
15. Pelham, H. R. B. *Cell* **46**, 959-961 (1986).
16. Pfanner, N., Tropschug, M. & Neupert, W. *Cell* **49**, 815-823 (1987).
17. Eilers, M., Opplinger, W. & Schatz, G. *EMBO J.* **6**, 1073-1077 (1987).
18. Chen, W.-J. & Douglas, M. G. *Cell* **49**, 651-658 (1987).
19. Eilers, M. & Schatz, G. *Nature* **322**, 228-232 (1986).
20. Rothman, J. E. & Kornberg, R. D. *Nature* **322**, 209-210 (1986).
21. Waters, M. G. & Blobel, G. *J. Cell Biol.* **102**, 1543-1550 (1986).
22. Hansen, W., Garcia, P. D. & Walter, P. *Cell* **45**, 397-406 (1986).
23. Rothblatt, J. & Meyer, D. I. *EMBO J.* **5**, 1031-1036 (1986).
24. Perara, E., Rothman, R. E. & Lingappa, V. R. *Science* **232**, 348-352 (1986).
25. Mueckler, M. & Lodish, H. F. *Nature* **322**, 549-552 (1986).
26. Zimmermann, R. & Mollay, C. *J. biol. Chem.* **261**, 12889-12895 (1986).
27. Craig, E. A. & Jacobsen, K. *Cell* **38**, 841-849 (1984).
28. Johnston, M. & Davis, R. W. *Molec. cell. Biol.* **4**, 2440-2448 (1984).
29. Julius, D., Schekman, R. & Thorner, J. *Cell* **36**, 309-318 (1984).
30. Deshaies, R. J. & Schekman, R. *J. Cell Biol.* **105**, 633-645 (1987).
31. Vassarotti, A., Chen, W.-J., Smagula, C. & Douglas, M. G. *J. biol. Chem.* **262**, 411-418 (1987).
32. Yaffe, M. P., Ohta, S. & Schatz, G. *EMBO J.* **4**, 2069-2074 (1985).
33. Yaffe, M. P. & Schatz, G. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4819-4823 (1984).
34. Waters, M. G., Chirico, W. J. & Blobel, G. *J. Cell Biol.* **103**, 2629-2636 (1986).
35. Pelham, H. R. B. *EMBO J.* **3**, 3095-3100 (1984).
36. O'Malley, K., Mauron, A., Barchas, J. D. & Kedes, L. *Molec. cell. Biol.* **5**, 3476-3483 (1985).
37. Verner, K. & Schatz, G. *EMBO J.* **6**, 2449-2456 (1987).
38. Weich, H., Sagstetter, M., Muller, G. & Zimmermann, R. *EMBO J.* **6**, 1011-1016 (1987).
39. Walter, P. & Lingappa, V. R. *A. Rev. Cell Biol.* **2**, 499-516 (1986).
40. Rothblatt, J. A., Webb, J. R., Ammerer, G. & Meyer, D. I. *EMBO J.* **6**, 3455-3463 (1987).
41. Crooke, E. & Wickner, W. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5216-5220 (1987).
42. Randall, L. L. & Hardy, S. J. S. *Cell* **46**, 921-928 (1986).
43. Ohta, S. & Schatz, G. *EMBO J.* **3**, 651-657 (1984).
44. Chen, W.-J. & Douglas, M. G. *J. biol. Chem.* **262**, 15598-15604 (1987).
45. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265-275 (1951).
46. Laemmli, U. K. *Nature* **227**, 680-685 (1970).
47. Burnette, W. N. *Anal. Biochem.* **112**, 195-203 (1981).
48. Emr, S. D., Vassarotti, A., Garrett, J., Geller, B. L., Takeda, M. & Douglas, M. G. *J. Cell Biol.* **102**, 523-533 (1986).
49. Chirico, W., Waters, G. & Blobel, G. *Nature* **332**, 805-810 (1988).

70K heat shock related proteins stimulate protein translocation into microsomes

William J. Chirico, M. Gerard Waters & Günter Blobel

Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

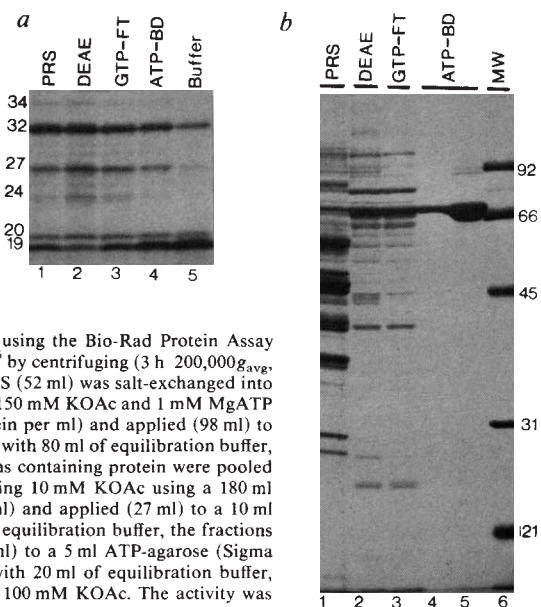
A yeast cytosol is shown to contain two distinct activities that stimulate protein translocation across microsomal membranes. One activity was purified. It consists of two constitutively expressed 70K heat shock related proteins that increase the rate of translocation. Possible mechanisms of action of these proteins are discussed.

EUKARYOTIC cells possess a number of protein translocation systems that permit translocation of specific proteins across distinct cellular membranes. Of these, the translocation system of the endoplasmic reticulum (ER) of higher eukaryotes is the best understood with regard to the number of components identified and characterized (for review see ref. 1). Much of this was accomplished using biochemical analyses of cell-free translocation systems. Cell-free systems reconstituting protein translocation across ER-derived microsomal membranes were developed recently using lower eukaryotes, such as *Saccharomyces cerevisiae*²⁻⁴ and *Neurospora crassa*⁵. The sophisticated genetic analyses that can be performed with these organisms,

combined with biochemical approaches, should allow a more complete description of the mechanism of protein translocation. However, counterparts of the cytosolic and membrane components of the ER translocation machinery that have been isolated from higher eukaryotic cells have not been identified in these lower eukaryotes. As a first step in this direction we demonstrated recently that *S. cerevisiae* cytosol contains an activity that stimulates translocation across yeast microsomal membranes⁶. Here we report that the activity consists of at least two distinct entities: one inactivated by N-ethylmaleimide (NEM), and one unaffected by NEM. Purification of the NEM-resistant activity shows that it consists of two closely related

Fig. 1 Purification of a translocation stimulating factor. **a**, Stimulation of post-translational translocation of ppaf into yeast microsomes by fractions obtained during purification from *S. cerevisiae* post-ribosomal supernatant (PRS). Translocation reactions were analysed using SDS-PAGE² and subsequent autoradiography. The bands correspond to untranslocated ppaf (19K), and translocated pro- α -factor (paf) (20K) having one (24K), two (27K) or three (32K) core oligosaccharides. Post-translational translocation assays⁶ were supplemented with various fractions (see methods) containing the indicated amounts of protein: lane 1, PRS (192 μ g), 70% translocation; lane 2, DEAE eluate (49.6 μ g), 85% translocation; lane 3, Flow-through from GTP-agarose column (22.4 μ g), 75% translocation; lane 4, Specifically-bound fraction from ATP-agarose column (4.8 μ g), 44% translocation; lane 5, Dialysis buffer alone, 22% translocation. **b**, Polypeptides in fractions obtained during the purification. Fractions containing the indicated amounts of protein were analysed using SDS-PAGE and stained with Coomassie Blue: lane 1, PRS (25 μ g); lane 2, DEAE eluate (15 μ g); lane 3, flow-through from GTP-agarose column, (15 μ g); lanes 4 and 5, specifically-bound fraction from ATP-agarose column (4 μ g and 20 μ g, respectively; lane 6, M_r markers, (Bio-Rad).

Methods. All purification steps were performed at 4°C. Protein concentrations were determined using the Bio-Rad Protein Assay which is a modification of the Bradford method²⁵. PRS (53 ml, 15 mg protein per ml) was prepared⁶ by centrifuging (3 h 200,000*g*_{avg}, Beckman Ti50.2 rotor), a cytoplasmic fraction (S100) derived from *S. cerevisiae* strain SKQ2N². PRS (52 ml) was salt-exchanged into Buffer A (20 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate, 2 mM dithiothreitol) containing 150 mM KOAc and 1 mM MgATP using a 1,000 ml Sephadex G-25 column. The excluded proteins were pooled (109 ml, 7.5 mg protein per ml) and applied (98 ml) to a 20 ml DEAE-cellulose (Whatman DE53) column equilibrated with the same buffer. After washing with 80 ml of equilibration buffer, the activity was eluted with Buffer A containing 300 mM KOAc and 1 mM MgATP. Eluate fractions containing protein were pooled (DEAE eluate: 15 ml, 3.9 mg protein per ml) and salt exchanged (14.4 ml) into Buffer A containing 10 mM KOAc using a 180 ml Sephadex G-25 column. The excluded protein fractions were pooled (28 ml, 2.1 mg protein per ml) and applied (27 ml) to a 10 ml GTP-agarose (Sigma, G9768) column equilibrated with the same buffer. After washing with 40 ml equilibration buffer, the fractions containing excluded protein were pooled (29.5 ml, 1.4 mg of protein per ml), and applied (28.3 ml) to a 5 ml ATP-agarose (Sigma A9264) column equilibrated with Buffer A containing 10 mM KOAc. The column was washed with 20 ml of equilibration buffer, then with 20 ml of Buffer A containing 1M KOAc, and finally with 20 ml of Buffer A containing 100 mM KOAc. The activity was specifically eluted with Buffer A containing 100 mM KOAc and 10 mM MgATP (8 ml, 0.4 mg protein per ml). GTP-agarose removed several polypeptides that would have copurified with the 70K protein if this step had been omitted. **a**, Samples were dialysed against Buffer A containing 100 mM KOAc and 1 mM MgATP and assayed for their ability to stimulate post-translational translocation as described⁶ except that the volume of the fraction to be analysed was 16 μ l and the 'compensation buffer' was adjusted to maintain the final ionic conditions of the assay⁶. GDP-mannose was included at a final concentration of 20 μ M, and prevented the formation of many of the partially glycosylated paf chains observed previously⁶. The percent translocation obtained from scanning gels directly for radioactivity (x) was adjusted to account for the loss of 1 of the 5 methionines in the precursor after signal sequence cleavage¹¹ [$125x/(0.25x+100)$]. The 19K protein was defined as untranslocated, while all products from 20 to 34K were defined as translocated¹¹.



proteins of relative molecular mass 70,000 (M_r 70K). Further characterization reveals that these 70K proteins are the products of the *SSA1* and *SSA2* genes, two constitutively expressed members of the 70K heat shock protein family^{7,8}. The implications of these findings for the general function of these proteins and their specific role in protein translocation are discussed.

A 70K cytosolic protein stimulates translocation

Protein translocation was assayed by translating messenger RNA coding for prepro- α -factor (ppaf)^{9,10} in a wheat germ translation system in the presence of [³⁵S]-methionine, inhibiting further translation with cycloheximide, and then incubating the resulting mixture with yeast microsomes, an ATP regenerating system, and samples to be tested for their ability to stimulate translocation. Analysis of reaction products using SDS-gel electrophoresis (SDS-PAGE) and subsequent autoradiography reveals essentially five bands corresponding to untranslocated ppaf (19K), and translocated pro- α -factor (paf) (20K) having one (24K), two (27K), or three (32K) core oligosaccharides^{2-4,11}. Paf migrates slower than ppaf in this SDS-PAGE system, although the signal sequence is removed during translocation¹¹.

The starting material for the purification of activity was a post-ribosomal supernatant (PRS) of *S. cerevisiae*⁶. Purification was monitored by assaying fractions for translocation activity (Fig. 1a and Table 1) and for polypeptide composition (Fig. 1b). In the absence of PRS ~22% of ppaf was translocated (Fig. 1a, lane 5). As previously reported⁶, PRS stimulated translocation (Fig. 1a and 1b, lane 1). When PRS was applied to a DEAE cellulose column at 150 mM salt the activity was retained. The activity was eluted by a 300 mM salt step (Fig. 1a and 1b, lane 2; Table 1). The 300 mM salt eluate from the DEAE column (referred to as DEAE eluate) was then chromatographed using nucleotide-agaroses, first on GTP-agarose and then on ATP-agarose. Most of the activity was recovered in the flow-through from GTP-agarose (Fig. 1a and 1b, lane 3; Table 1). The GTP flow-through was then applied to ATP-agarose. After washing

in 1 M salt, about 20% of the total activity present in the GTP-agarose flow-through was specifically eluted by ATP (Fig. 1a and 1b, lane 4; Table 1). The ATP-eluted fraction consisted primarily of a 70K polypeptide (Fig. 1b, lanes 4 and 5). The genetic data of Deshaies *et al.*¹² (see below) suggest that this 70K protein, rather than the other minor polypeptides (Fig. 1b, lane 5), is responsible for the observed stimulation of translocation.

Two distinct translocation-stimulating activities

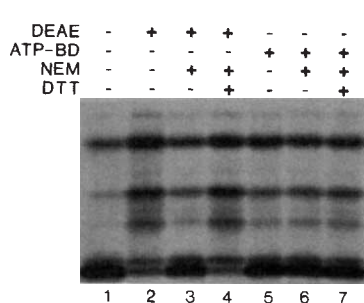
Previously we showed that the translocation stimulating activity in the PRS is inactivated by the sulfhydryl alkylating reagent (NEM)⁶. To determine whether the purified 70K protein is the NEM-sensitive component we compared the activity of the DEAE eluate and the 70K protein with or without NEM pretreatment. When the DEAE eluate was pretreated with NEM

Table 1 Purification of translocation stimulating activity

Step	Total protein (mg)	Total activity (U)*	Specific activity (U mg ⁻¹)	Purification (x-fold)	Activity recovery (%)
PRS	795.0	451,000	567	1.0	100.0
DEAE	66.7	400,000	6,000	10.6	88.7
GTP-FT	51.0	299,000	5,860	10.3	66.3
ATP-BD	4.1	64,800	15,800	27.9	14.4

* We define one unit (U) of activity as 1% translocation above background of ppaf into yeast microsomes under standard conditions (see Fig. 1 legend). The activity in 1, 2, 4, 8 and 16 μ l of each protein fraction obtained during successive purification steps was assayed (see Fig. 1 legend) and the percent translocation was determined (see Fig. 1 legend). A linear regression performed on data in the linear range (<43% translocation) gave the slope (% translocation per ml) which was divided by the protein concentration (mg ml⁻¹) of the fraction to yield the specific activity (U mg⁻¹). Yields were corrected for sampling during purification.

Fig. 2 NEM inhibits the activity of the DEAE eluate but not of the purified 70K protein. Post-translational translocation assays were supplemented with either DEAE eluate containing the amounts of protein indicated, or purified 70K protein (ATP-BD), pretreated



without or with NEM: lane 1, Buffer A containing 100 mM KOAc and 1 mM MgATP (35% translocation); lane 2, DEAE eluate (45 µg), 81% translocation; lane 3, DEAE eluate (45 µg) treated with NEM, 42% translocation; lane 4, DEAE eluate (45 µg) treated with NEM and DTT, 90% translocation; lane 5, purified 70K protein, (4.4 µg), 57% translocation; lane 6, purified 70K protein (4.4 µg) treated with NEM, 56% translocation; lane 7, purified 70K protein (4.4 µg) treated with NEM and DTT, 58% translocation. The 34K protein evident in this autoradiograph is triply glycosylated paf which has untrimmed oligosaccharides¹¹.

Methods. Protein fractions were dialysed against Buffer A containing 100 mM KOAc and 1 mM MgATP. Aliquots were then incubated at 20 °C for 15 min with 1/10 volume of water (lanes 2 and 5), or with a final concentration of 10 mM NEM (lanes 3 and 6), or with a mixture of 10 mM NEM and 20 mM DTT that was preincubated at 0 °C 10 min (lanes 4 and 7). The reactions were cooled on ice and a final concentration of 20 mM DTT was used to inactivate unreacted NEM in NEM-treated samples (lanes 3 and 6). The resulting mixtures were incubated for 10 min at 0 °C before samples (16 µl) were used in post-translational translocation assays as described in Fig. 1. Products of reactions were analysed using SDS-PAGE² and subsequent autoradiography. Percent translocation was determined as described in Fig. 1.

its activity was largely abolished (Fig. 2, lanes 1–4) whereas pretreatment of the 70K protein with NEM had no effect on its activity (Fig. 2, lanes 1 and 5–7). These data suggest that the DEAE eluate consisted of at least two distinct activities, one inactivated by NEM and the other insensitive to NEM. The 70K protein represents the NEM-insensitive activity. Both activities are required for efficient translocation, because the 70K protein alone, even when added in saturating amounts, only yielded about 50–60% translocation, whereas cruder fractions yielded about 90% translocation (data not shown). The fact that the NEM-sensitive factor, present in crude fractions but not in the 70K protein preparation, is required for full activity means that the estimated purification of the 70K protein based on specific activity (Table 1) may represent a significant underestimate of the actual purification achieved.

Characteristics of purified 70K protein

Purified 70K protein binds ATP. The specific elution of the 70K protein from ATP-agarose suggested that it contains an ATP binding site. We confirmed this by photoaffinity crosslinking using [α -³²P]-ATP (Fig. 3)¹³. Labelling was dependent on UV radiation (Fig. 3, lanes 1 and 2) and was strongly inhibited by 0.01 or 0.1 mM ATP (Fig. 3, lanes 3 and 4). GTP also inhibited the labelling, but 100-fold higher concentrations were required (Fig. 3, lanes 5 and 6). This is consistent with the weak binding of the 70K protein to GTP-agarose during the purification (data not shown).

70K protein is a heat shock protein. The molecular mass and the ability to bind ATP suggested that the purified protein might be a member of the 70K heat shock protein (hsp70) family^{8,14}. Indeed, a monoclonal antibody raised against *Drosophila* hsp70 that recognizes an evolutionarily conserved epitope of members of the hsp70 family¹⁵, reacted on a Western blot (Fig. 4a) with the purified 70K protein (lane 3). A 70K species, presumably identical to the purified 70K protein, was the only reactive protein of yeast PRS (lane 2). Purified heat shock related proteins from HeLa cells¹⁶ were also recognized (lane 1).

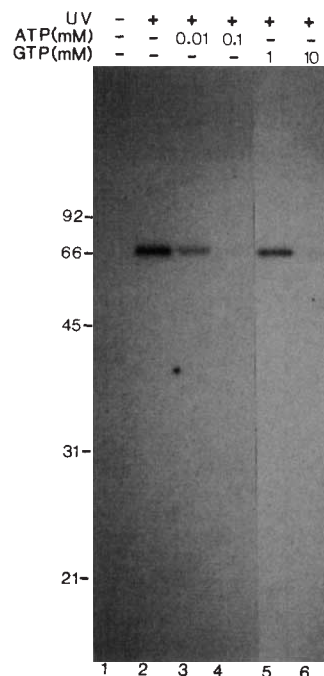


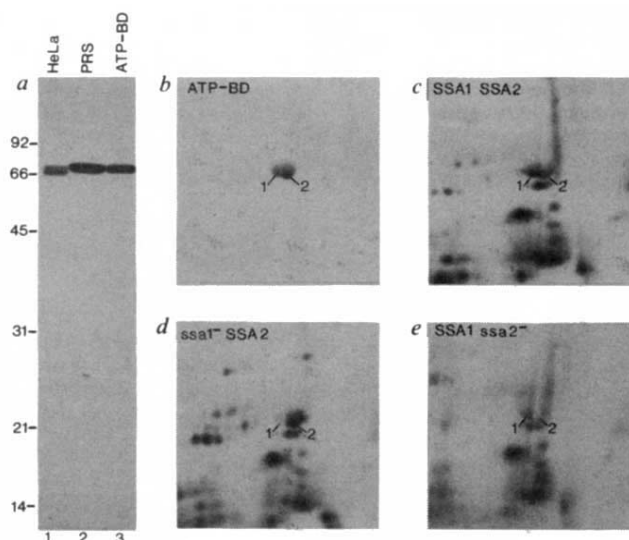
Fig. 3 Photoaffinity crosslinking of purified 70K protein with [α -³²P]ATP. Each lane represents the products of a reaction containing purified 70K protein and [α -³²P]ATP either without UV irradiation (lane 1), or with UV irradiation in the absence of ATP (lane 2) or in the presence of 0.01 mM ATP (lane 3), 0.1 mM ATP (lane 4), 1.0 mM GTP (lane 5), or 10.0 mM GTP (lane 6).

Methods. Each reaction contained [α -³²P]ATP (10 µCi, 3000 Ci mmol⁻¹) and purified 70K protein (2.5 µg) that had been dialysed against 20 mM sodium phosphate, 100 mM KOAc, 2 mM Mg(OAc)₂, 2 mM DTT in a final volume of 20 µl. Samples were incubated at 0 °C for 15 min and then photolysed for 30 min using a 254 nm lamp (Model UVS-11 Ultra-violet Products) at a distance of 2 cm. Products of crosslinking reactions were separated using SDS-PAGE². Gels were fixed, dried, and then exposed to Kodak XAR-5 film using an intensifying screen at -80 °C.

S. cerevisiae contains at least eight genes coding for members of the hsp70 family of which six are constitutively expressed^{17,8}. To determine which gene product we had purified, we electrophoresed the 70K protein in two dimensions (isoelectric focusing in the first dimension and SDS-PAGE in the second dimension). The purified protein was resolved into two species having isoelectric points of about 5.4 and 5.5 (Fig. 4b). Both proteins were recognized by the monoclonal antibody raised against *Drosophila* hsp70 (data not shown). The two-dimensional electrophoretic pattern¹⁷ suggested that the two proteins were the gene products of *SSA1* and *SSA2* (referred to as Ssa1p and Ssa2p, respectively). To investigate this, the 70K proteins, as internal standards, were mixed with [³⁵S]-methionine-labelled cell lysates from a wild-type strain, an *ssa1*⁻ strain, or an *ssa2*⁻ strain, and then each mixture was electrophoresed in two dimensions. We detected the purified proteins using Coomassie Blue and the [³⁵S]-methionine-labelled proteins using autoradiography. The wild-type strain had both proteins (Fig. 4c), the *ssa1*⁻ strain lacked the protein with pI of 5.5 (Fig. 4d), and the *ssa2*⁻ strain lacked the protein with a pI of 5.4 (Fig. 4e). These results indicate that purified 70K protein consists of two species, Ssa1p and Ssa2p, which are 98% homologous (E. Craig, personal communication). The absence of a phenotypic effect in either *ssa1*⁻ or *ssa2*⁻ mutants, but the presence of a phenotypic effect in *ssa1*⁻/*ssa2*⁻ double mutants, suggest that these proteins are functionally similar¹⁸. **Ssa1p/Ssa2p increases translocation rate.** To determine whether Ssa1p/Ssa2p (that is, purified 70K protein) affected the rate of translocation of paf, we analysed the time course of translocation (Fig. 5). The initial rate of translocation was ~eightfold

Fig. 4 Purified 70K protein is immunologically related to hsp70 and consists of two closely related proteins, Ssa1p and Ssa2p. *a*, Western blot of proteins recognized by a rat monoclonal antibody raised against *Drosophila* hsp70: 2.5 μ g of purified HeLa hsp 73/72K (HeLa, lane 1), 100 μ g of yeast PRS (lane 2), and 2.5 μ g of purified 70K protein eluted from the ATP-agarose column (ATP-BD, lane 3). *b*, Two-dimensional electrophoresis of purified 70K protein detected using Coomassie Blue. *c-e*, Mixtures of 4 μ g of purified 70K protein and proteins from [35 S]Met-labelled cell lysates from a wild-type strain (*c*), an *ssa1⁻* strain (*d*) or an *ssa2⁻* strain (*e*) detected using autoradiography.

Methods. *a*, Proteins were separated using SDS-PAGE as described² and then blotted electrophoretically at 4°C on to nitrocellulose paper (Schleicher and Schuell, 0.45 μ m) in 20 mM Tris, 150 mM glycine, 20% methanol, and 0.01% SDS at 40 V for 4 h. The blot was incubated with 3% bovine serum albumin (BSA), 20 mM sodium phosphate, pH 7.5, 140 mM NaCl for 2 h at room temperature, washed with water and then incubated with monoclonal antibody 7.10 raised against *Drosophila* hsp70¹⁵ at a 1:400 dilution in Buffer W (1% BSA, 20 mM sodium phosphate, 140 mM NaCl, 0.1% Triton X-100, and 0.02% SDS) for 2 h. After extensively washing with Buffer W, the blot was incubated with rabbit anti-mouse IgG (Cooper Biomedical) at a 1:1000 dilution in Buffer W for 2 h. Further extensive washing with Buffer W was followed by incubation with 125 I-labelled protein A (9 μ Ci μ g⁻¹, 118 μ Ci ml⁻¹, New England Nuclear) at a 1:2500 dilution in Buffer W for 2 h. After washing the blot with Buffer W, the blot was dried and exposed to Kodak XAR-5 film with an intensifying screen. We used rabbit anti-mouse IgG, which also recognizes the rat monoclonal, instead of rabbit anti-rat IgG as the second antibody, because the rabbit anti-rat IgG recognized several proteins in yeast PRS. Neither rabbit anti-mouse IgG nor 125 I-labelled protein A alone recognized the blotted proteins (data not shown). *b*, Purified 70K protein (4 μ g) was electrophoresed in two dimensions (isoelectric focusing in the first dimension and SDS-PAGE in the second dimension) as described.¹⁷ *c-e*, Yeast strains (T127: *leu2-3, 112, ade2-107, Δ trp1, ura3-52, lys2, MATa*; T125: *his4-173, Δ trp1, ura3-52, lys2[SSA1:leu2] leu2-3, 112, MATa*; T126: *Δ trp1, ura3-52, lys2, leu2-3, 112, [SSA2:leu2]MATa*) were grown at 30°C to an absorbance at 600 nm of one unit per ml in SD media (0.67% Bacto yeast nitrogen base without amino acids, 2% dextrose)³⁶ supplemented with 20 μ g ml⁻¹ each of adenine, uracil, histidine-HCl and tryptophan and 30 μ g ml⁻¹ of leucine and lysine-HCl. Samples (2.5 ml) of each culture were transferred to separate glass tubes, the cells collected by centrifugation, washed with an equal volume of growth media, and resuspended in 1.25 ml of 30°C growth media. Each culture was incubated with 25 μ l of [35 S]methionine (New England Nuclear, 10 Ci ml⁻¹) at 30°C for 30 min. Trichloroacetic acid (TCA) was added to 5% and the cultures incubated at room temperature for 15 min. Cell precipitates were collected by centrifugation at 2500g for 10 min, and resuspended in 150 μ l of 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1% SDS. Glass beads (100 mg Sigma, Type V; washed in 1 N HCl) were added, the tubes were vortexed vigorously for 2 min and incubated at 100°C for 3 min. The lysates were diluted by adding 1 ml of 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween 20, 1 mM phenylmethylsulfonyl fluoride, mixed and centrifuged as above. The supernatants were collected and TCA precipitable counts were determined. A sample (2 $\times$ 10⁶ c.p.m.) of each lysate was mixed with 4 μ g of purified 70K protein and then precipitated in 10% TCA before resuspension for two-dimensional electrophoresis.



higher in the presence of Ssa1p/Ssa2p than in its absence (Fig. 5c, curves N and N+S).

Urea denaturation increases translocation rate. The rate of *in vitro* protein import into mitochondria can be dramatically increased upon denaturation of the precursor by urea prior to import (Eilers and Schatz, personal communication). To investigate whether urea denaturation of ppaf would increase the rate of translocation across yeast microsomal membranes we performed a similar experiment. We diluted a translation mixture containing ppaf with three volumes of either water (control) or 8 M urea (yielding a final concentration of 6 M urea) and preincubated the resulting mixture for 5 min at 20°C. Translocation was initiated by dilution of the mixture into translocation reactions containing either Ssa1p/Ssa2p or buffer. The final concentration of urea was 100 mM. The initial rate of translocation of urea-denatured ppaf was about 11-fold higher (Fig. 5c, curve D) than that of native ppaf (Fig. 5c, curve N) suggesting that unfolding (or disaggregation) of ppaf was a rate limiting step. An even faster initial rate of translocation (about 19-fold higher than curve N) was observed when ppaf was denatured and then translocated in the presence of Ssa1p/Ssa2p (Fig. 5c, curve D+S) suggesting that the refolding of ppaf after urea dilution might be slowed by Ssa1p/Ssa2p or that Ssa1p/Ssa2p might catalyse unfolding (see below). The time courses of control reactions where ppaf was preincubated with water, but where translocation reactions contained 100 mM urea, were indistinguishable from those of reactions that lacked urea (data not shown).

Conclusions

We have shown that yeast cytosol contains two distinct activities both of which are required for efficient post-translational trans-

location of ppaf across yeast microsomal membranes. One of these activities was demonstrated to be NEM-sensitive, whereas the other was shown to be NEM-resistant. The NEM-resistant activity co-purified with a 70K protein that specifically bound ATP. The purified 70K protein reacted with a monoclonal antibody that recognizes an evolutionarily conserved epitope of 70K heat shock proteins. Two-dimensional electrophoresis revealed that it consisted of two constitutively expressed 70K heat shock related proteins, the SSA1 and SSA2 gene products. The 98% homology of Ssa1p and Ssa2p (E. Craig, personal communication), and the phenotypes of mutants in either one or both genes¹⁸, suggest that they are functionally similar. We showed that Ssa1p/Ssa2p increases the rate of translocation. Furthermore, the rate of translocation was increased when ppaf was denatured by urea prior to translocation suggesting that the conformation of ppaf influences its rate of translocation.

The mechanism by which Ssa1p/Ssa2p affects the rate of translocation remains to be elucidated. These two proteins may be the yeast equivalent of 'uncoating ATPase,' a heat shock related protein¹⁷, previously isolated from bovine brain¹⁹. A hypothesis that proposes a general function for heat shock related proteins and that might explain their role in clathrin uncoating, and perhaps other cellular processes, was formulated recently by Pelham²⁰. In this scheme, hsps bind tightly to hydrophobic sites exposed in unfolded or aggregated proteins. Using the energy of ATP hydrolysis, hsps undergo a conformational change before or during release from the substrate. The conformational change in the hsp may 'distort' the substrate, and by repeated cycles may catalyse refolding or disaggregation of proteins.

Ssa1p/Ssa2p might increase the rate of translocation in several ways that are consistent with Pelham's²⁰ proposal. If ppaf was

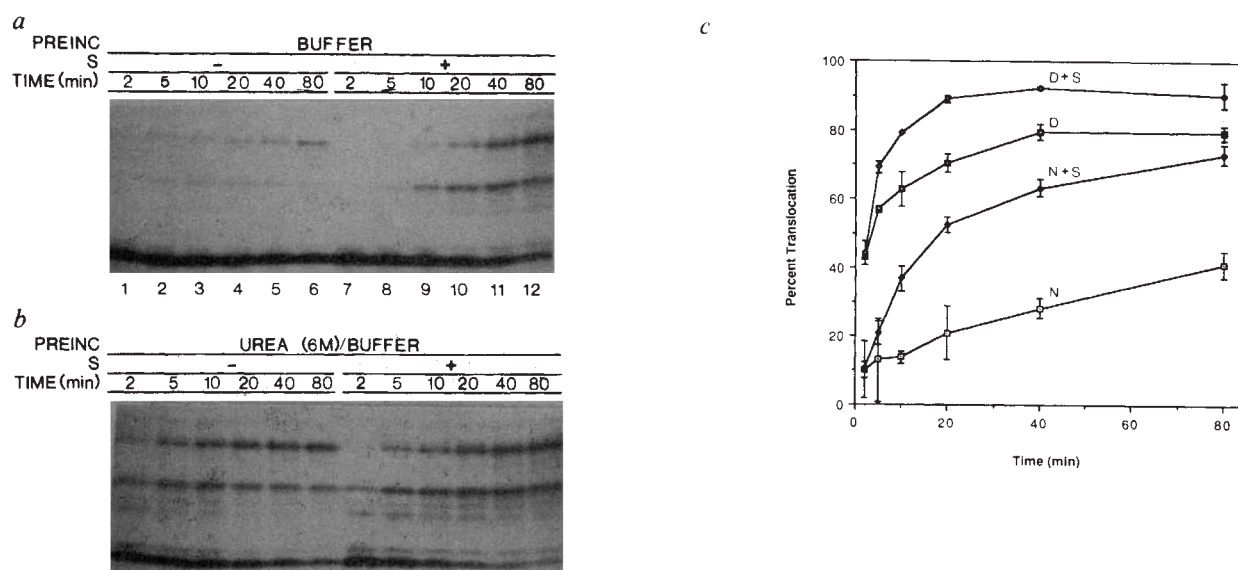


Fig. 5 Effect of urea denaturation of ppaf on the rate of translocation in the absence or presence of Ssa1p/Ssa2p. *a*, Kinetics of translocation of native ppaf in the absence (lanes 1-6: 2, 5, 10, 20, 40 and 80 min, respectively) or presence (lanes 7-12: 2, 5, 10, 20, 40 and 80 min, respectively) of Ssa1p/Ssa2p. *b*, As in *a* except with urea-denatured ppaf. *c*, Percent translocation of reactions in *a* and *b* was quantitated (see legend to Fig. 1) for three separate experiments and the mean $\pm$ the standard deviation was plotted as a function of time. Assays contained either native ppaf (N) or urea-denatured ppaf (D), supplemented without or with Ssa1p/Ssa2p (S).

Methods. After translation of ppaf in a wheat germ system, the reaction was diluted fourfold with either water (*a*) or 8 M urea (*b*) and then preincubated for 5 min at 20 °C. Other constituents of the post-translational translocation assays were as described⁶ except that each was supplemented with 5.7 μ l of wheat germ buffer to maintain the described ionic conditions and assays received either 1.2 μ l 6 M urea (*a*) or 1.2 μ l water to ensure a final urea concentration of 100 mM in all translocation reactions. Half the post-translational translocation assays (lanes 7-12) were also supplemented with 14.4 μ g of Ssa1p/Ssa2p. The post-translational translocation assays were preincubated for 5 min at 20 °C, before initiating the translocation reactions by the addition of 1.2 μ l native or denatured ppaf. Reactions were incubated at 20 °C, and aliquots (10 μ l) were removed at the indicated times and then prepared for SDS-PAGE². Gels were fixed, dried, and autoradiographed. Control reactions without urea were indistinguishable from those that contained 100 mM urea (data not shown). Rates of translocation (% of ppaf translocated per min) were estimated from early, linear portion of time courses.

present in an aggregated form after translation, Ssa1p/Ssa2p could disperse these aggregates. Alternatively, if individual ppaf molecules folded into a translocation-incompetent conformation^{21,22,27-30} Ssa1p/Ssa2p might unfold ppaf before or during translocation.

In vivo, ppaf is probably translocated during translation, therefore unfolding of a completed protein or disaggregation may not be required. Nevertheless, Deshaies *et al.*¹² showed that Ssa1p affects translocation *in vivo*. Therefore, Ssa1p/Ssa2p may salvage precursors that prematurely fold into translocation-incompetent conformations during translation. However, mechanisms other than unfolding or disaggregation of the substrate should be considered. We envision that the conformational change of Ssa1p/Ssa2p could be used to hide its 'active' (that is, surface exposed) hydrophobic site³¹. In this inactive conformation, Ssa1p/Ssa2p would no longer be able to bind to an exposed hydrophobic site of another protein. Only after rebinding of ATP would the hydrophobic site of Ssa1p/Ssa2p be 'reactivated.' The 'active' hydrophobic site of Ssa1p/Ssa2p could then interact with another Ssa1p/Ssa2p to form a homodimer or with an exposed hydrophobic site of another protein to form a heterodimer. Many cellular proteins may contain exposed hydrophobic sites that are functionally important. The binding cycles of Ssa1p/Ssa2p would function reversibly to 'protect' and 'deprotect' active hydrophobic sites.

Ssa1p/Ssa2p may function via this mechanism on several levels that are not mutually exclusive. For example, it could interact directly with the hydrophobic signal sequence, preventing it from folding up with the rest of the chain, and thereby maintain translocation competence. However, unlike signal recognition particle (SRP)²³ (or, its yeast equivalent) Ssa1p/Ssa2p

may not possess a targeting function. If SRP-mediated targeting is required, an interaction of the signal peptide with Ssa1p/Ssa2p would not be directly productive. Another possibility is that Ssa1p/Ssa2p is involved in the protection/deprotection of an exposed hydrophobic site of SRP that interacts with the signal peptide. Or, Ssa1p/Ssa2p may be involved in the protection/deprotection of an exposed hydrophobic site of the signal sequence receptor²⁴ in the membrane, or other unidentified translocation components.

Ssa1p/Ssa2p represents a cytosolic factor involved in protein translocation that is distinct from SRP. The NEM-sensitive activity, which we are purifying, may possess a targeting function similar to that of SRP.

We thank Susan Lindquist and Hugh Pelham for monoclonal antibody (7.10), William Welch for purified heat shock proteins (hsp73/72) from HeLa cells, Elizabeth Craig for *ssa1*⁻ and *ssa2*⁻ yeast strains, and Ray Deshaies, B. D. Koch, Randy Schekman, Margaret Werner-Washburne and Elizabeth Craig for sharing results before publication. We thank Jacques YaDeau, Gary Greenburg and John Aris for helpful discussions. We especially acknowledge discussions with Rubén Henríquez and thank him for his initial Western blot.

Received 2 March; accepted 14 March 1988.

1. Walter, P. & Lingappa, V. R. *A. Rev. Cell Biol.* **2**, 499-516 (1986).
2. Waters, M. G. & Blobel, G. *J. Cell Biol.* **102**, 1543-1550 (1986).
3. Hansen, W., Garcia, P. D. & Walter, P. *Cell* **45**, 397-406 (1986).
4. Rothblatt, J. A. & Meyer, D. I. *Cell* **44**, 629-637 (1986).
5. Addison, R. *J. Biol. Chem.* **262**, 17031-17037 (1987).
6. Waters, M. G., Chirico, W. J. & Blobel, G. *J. Cell Biol.* **103**, 2629-2636 (1986).
7. Ingolia, T. D., Slater, M. R. & Craig, E. A. *Molec. Cell Biol.* **2**, 1388-1398 (1982).
8. Craig, E. A. *CRC Crit. Rev. Biochem.* **18**, 239-280 (1985).
9. Kurjan, J. & Herskowitz, I. *Cell* **30**, 933-943 (1982).

10. Sprague, Jr, G. F., Blair, L. & Thorner, J. A. *Rev. Microbiol.* **37**, 623-660 (1983).
11. Waters, M. G., Evans, E. & Blobel, G. *J. biol. Chem.* (in the press).
12. Deshaies, R., Koch, B. D., Werner-Washburne, M., Craig, E. & Schekman, R. *Nature* (in the press).
13. Yue, V. T. & Schimmel, P. R. *Biochemistry* **16**, 4678-4684 (1977).
14. Lindquist, S. A. *Rev. Biochem.* **55**, 1151-1191 (1986).
15. Kurtz, S., Rossi, J., Petko, L. & Lindquist, S. *Science* **231**, 1154-1157 (1982).
16. Welch, W. J. & Feramisco, J. R. *J. biol. Chem.* **257**, 14949-14959.
17. Chappell, T. G., Welch, W. J., Schlossman, D. M., Palter, K. B., Schlesinger, M. J. & Rothman, J. E. *Cell* **45**, 3-13 (1986).
18. Craig, E. A. & Jacobsen, K. *Cell* **38**, 841-849 (1984).
19. Schlossman, D. M., Schmid, S. L., Braell, W. A. & Rothman, J. E. *J. Cell Biol.* **99**, 723-733 (1984).
20. Pelham, H. R. B. *Cell* **46**, 959-961 (1986).
21. Eilers, M. & Schatz, G. *Nature* **322**, 228-232 (1986).
22. Randall, L. L. & Hardy, S. J. S. *Cell* **46**, 921-928 (1986).
23. Walter, P. & Blobel, G. *Nature* **299**, 691-698 (1982).
24. Wiedmann, M., Kurschalia, T. V., Hartmann, E. & Rapoport, T. A. *Nature* **328**, 830-833 (1987).
25. Bradford, M. M. *Analyt. Biochem.* **72**, 248-254 (1976).
26. Hicks, J. B. & Herskowitz, I. *Genetics* **83**, 245-258 (1976).
27. Rothman, J. E. & Kornberg, R. D. *Nature* **322**, 209-210 (1986).
28. Pfanner, N., Tropschug, M. & Neupert, W. *Cell* **49**, 815-823 (1987).
29. Crooke, E. & Wickner, W. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5216-5220 (1987).
30. Chen, W. & Douglas, M. G. *J. biol. Chem.* **262**, 15598-15604 (1987).
31. Guidon, P. T., Jr & Hightower, L. E. *Biochemistry* **25**, 3231-3239 (1986).

LETTERS TO NATURE

Solar luminosity variations in solar cycle 21

Richard C. Willson* & H. S. Hudson†

* Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91109, USA

† Center for Astrophysics and Space Science, University of California at San Diego, La Jolla, California 92093, USA

The ACRIM I experiment (Active Cavity Radiometer Irradiance Monitor) on the solar maximum Mission (SMM) satellite has provided a nearly continuous record of solar total irradiance variations since early 1980¹. It has detected variations on time scales ranging from minutes to SMM's lifetime. The long-term variations have revealed a downward trend during the declining phase of solar cycle 21 (ref. 2) of the sunspot cycle, a flat period between mid-1985 and mid-1987, and an upturn in late 1987 which suggests a direct correlation of luminosity and solar active region population. If the upturn continues into the activity maximum of solar cycle 22, a relation between solar activity and luminosity of possible climatological significance could have been discovered. The sense of the correlation agrees with what has been predicted from the coincidence of the 'little ice age' climate anomaly in the sixteenth and seventeenth centuries and the Maunder Minimum of solar activity³. The best-fit relationship for the variation of total irradiance S , with sunspot number R_z , and 10-cm flux F_{10} , are $S = 1,366.82 + 7.71 \times 10^{-3} R_z$ and $S = 1,366.27 + 8.98 \times 10^{-3} F_{10}$ (W m^{-2}). These could be used to approximate total irradiance variations over the periods for which these indices have been compiled.

ACRIM I consists of three independent, electrically self-calibrating cavity radiometers, which detect solar irradiance over a spectral range from far ultraviolet to far infrared wavelengths with uniformly high efficiency. The three radiometers are operated with different observing frequencies: one monitors the sun continuously, the second calibrates the degradation of the first about once per month, and the third is occasionally used in comparison with the first two as an ultimate degradation control experiment. Each sensor has a shutter with a 131.072-s period. This internal system for monitoring detector performance, plus cross-references against independent Nimbus-7 observations⁴ and rocket and balloon calibrations has established the reliability of these measurements even on the longest time scales. The relative error, as determined by the independent measurements on board SMM, is of the order of 20 parts per million².

Results from 1980 to late 1987 are shown in Fig. 1 in the form of the mean of all observations for each day for which observations were obtained. The record is divided into several distinct periods of data quality. The first is from two days after launch in February 1980 (1980 day 47) through 1980 day 346, during

which time SMM used its fully precise, three-axis solar pointing. The attitude control system failed in late 1980 and SMM was put into a spin-stabilized mode. Because the spacecraft was not designed for spin stabilization, the result was a 'wobbly' 10° cone about the solar line-of-sight. The solar disk passed through ACRIM I's $\pm 1^\circ$ field-of-view, often enough to provide an average of 100 sets of observations per day, but both the quality and quantity of data acquisition were significantly reduced from the 'precision solar-pointed' mode². The third and present period, following repair of the SMM satellite's solar pointing subsystem by the space shuttle astronauts in March 1984, has the same high data acquisition frequency and quality as the initial year's measurements.

Although the general features of solar total irradiance modulation by solar activity are accessible during all three periods of SMM/ACRIM I operation, the highest quality data are from the two 'precision solar-pointed' periods. For this reason, and because these two periods represent distinctly different states of solar activity, the correlation with sunspot number and 10-cm flux discussed below use only the pointed-mode data.

In the two 'precision solar-pointed' periods, the dominant sources of variability in the data are of solar origin. In the spin mode, ACRIM I's rate of data acquisition was reduced by a factor of more than 100, with the result that the individual sampling noise level ($\pm 0.02\%$) occasionally exceeded the day-to-day solar variations. Additionally, a 0.12% scale shift of the results was detected, caused by the altered mode of sensor operation during the period. It provided an additional source of uncertainty that further reduced the quality of spin mode results². (The scale shift was calibrated using the 1980 'precision solar-pointed' data with a residual uncertainty of $\pm 0.02\%$. Its effect has been removed from the results shown in Fig. 1.)

The abrupt change in the fluctuation in the results of Fig. 1, in early 1984, is partly in artefact of the transition from spin mode to precision pointed mode. An additional component of apparent day-to-day variability was in fact a measurement uncertainty introduced into the results by the spin mode. Its magnitude can be estimated by comparing the results from 1980 and after mid-1984. The differences detectable on this scale are entirely solar in origin, reflecting the difference in solar activity levels. Had the SMM pointing system not failed, a more gradual transition in variability of the database from the 1980-81 solar activity peak to the 1986 solar activity minimum would have been recorded.

A cosine curve shown in Fig. 1 has been fitted to the ACRIM I results to the apparent solar cycle dependence of the irradiance. This curve has an assumed period of 10.95 years (4,000 days) and a maximum epoch of 1980.82. The amplitude of the least-mean-square fit is $0.039 \pm 0.003\%$ of the average total irradiance ($S_p = 1367.72 \text{ W m}^{-2}$). As can be seen in Fig. 1, the cosine is a good approximation to the 1985-87 results, including the period around magnetic solar minimum in August 1986. The poorer fit in 1980 and during the spin mode makes it clear that this

Fig. 1 ACRIM I daily mean data from early 1980 to late 1987. The dashed vertical lines separate the two 'precision solar pointed' periods (most of 1980 and after March 1984) from the intervening 'spin-mode' period. The dashed curve is a fitted least-mean-square cosine function,

$$S = S_0(1 + 0.039$$

$$\times \cos(2\pi(t - 1980.82)/10.95));$$

period 4,000 d; offset 300 d; amplitude $0.039 \pm 0.003\%$.

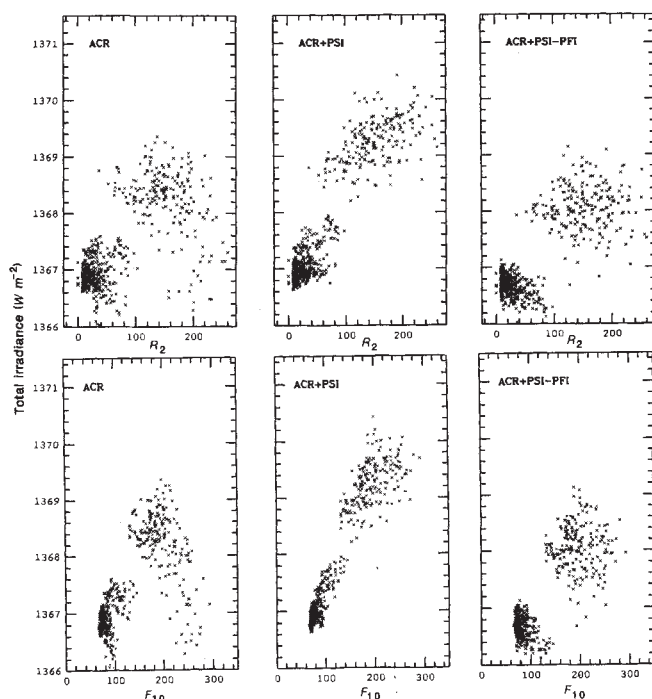
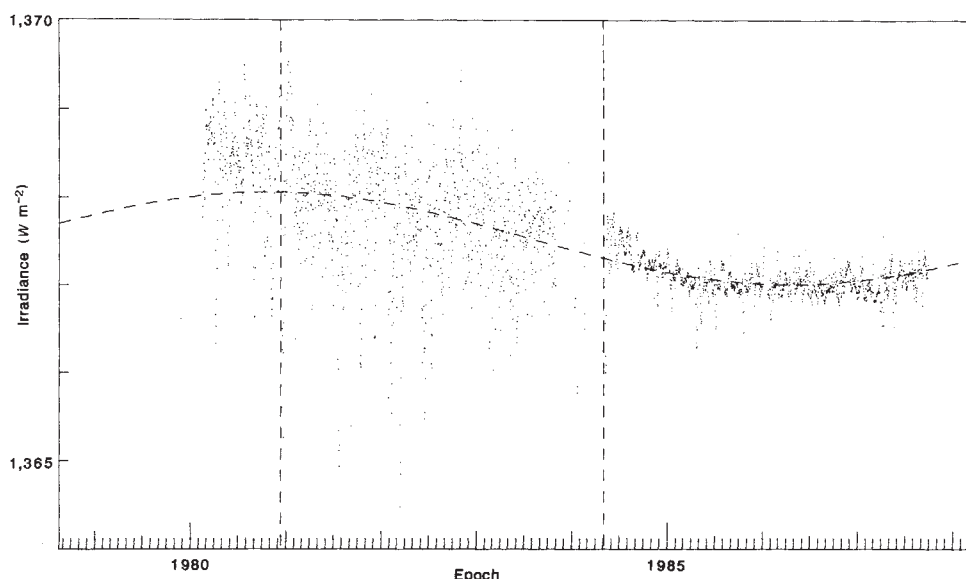


Fig. 2 Correlation of ACRIM I mean daily data against the Zürich sunspot number (a) and solar 10-cm radio flux (b). Left panels: original data; middle panels: results adjusted for sunspot flux deficit (PSI); right panels: results adjusted for both PSI and facular flux excess (PFI). The two groupings derive from the two periods of 'precision solar pointing'; the 1980 group has generally higher flux and solar indices values.

approximation to the luminosity time series is too simplistic, and that multiple modes of solar luminosity/activity relationships may be required to explain the cycle with precision.

On active-region time scales (days to months), sunspots and active-region faculae have been found to cause much of the variability detected by the ACRIM I experiment. The dominant irradiance effects are decreases due to sunspots, but effects due to active-region faculae are also easily observed in the residuals resulting from simple corrections for the sunspot effects⁵⁻⁷. Modulation of total irradiance by faculae associated with solar active regions, and the approximate balancing between sunspot

radiative deficit and active region facular excess on time scales up to a year, have been cited and discussed previously⁶⁻¹². This paper explores the possibility that the long-term trend in the ACRIM I results is caused by a solar activity cycle modulation of the photospheric brightness by a white-light, facular-type irradiance component both within and outside active regions.

We have used simple models to estimate the magnitudes of solar active region sunspot and facular irradiance effects in an effort to remove them, to first order, from the irradiance record, and facilitate a clearer understanding of the longer-term irradiance variability. The models use synoptic sunspot and Ca-plage data to estimate the 'sunspot deficit' (photometric sunspot index, PSI) and 'facular excess' (photometric facular index, PFI) irradiance components on a day-by-day basis. The models are

$$\text{PSI:} \quad A \sum_i S_i \mu_i (3\mu_i + 2)/2$$

$$\text{PFI:} \quad B \sum_i P_i \mu_i g_i (3\mu_i + 2)/2$$

where μ_i is the cosine of active region distance to disk centre, P_i is sunspot area (from Solar Geophysical Data), S_i is plage area (from Solar Geophysical Data), so $S_i \mu_i$ and $P_i \mu_i$ are sunspot and plage projected areas. In addition, $(3\mu_i + 2)/2$ is the photospheric limb-darkening function, g_i is the empirical facular contrast function¹⁰, $A = 3.5 \times 10^{-7}$ is the irradiance conversion factor for PSI (ref. 9) and $B = 1.2 \times 10^{-6}$ is the irradiance conversion factor for PFI (ref. 8).

The PSI and PFI models use the daily values of sunspot and Ca-plage areas published in the NOAA Solar Geophysical Data. We investigate the success with which their inclusion reduces the variance in the ACRIM I data base and explore the resulting modified ACRIM I results for relationships with the solar cycle irradiance trend.

The first step in this process is demonstrated in Fig. 2, which shows the correlations of the original ACRIM I results, the PSI adjusted results and the results adjusted for both PSI and PFI. Figure 2 shows the dependence separately for the Zürich sunspot number and the 10-cm radio flux. The scatter downward and to the right in the original 1980 results (upper grouping of data in all frames) is caused by the frequent occurrences of large sunspot groups in that epoch. The tighter grouping and smaller range of irradiance and solar activity indices for the 1984-86 results (lower left of all frames) reflects the lower incidence of solar activity during that period.

The second panels of Fig. 2 illustrate the success of the PSI model in reducing the irradiance variation caused by sunspots. A substantial reduction in the scatter of results and a net increase

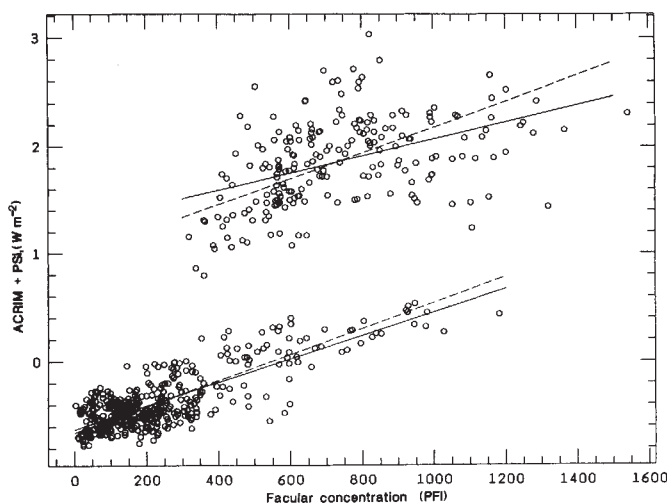


Fig. 3 Correlation of the sunspot-adjusted data (ACRIM I + PSI) with the modelled facular irradiance contributions (PFI). Dashed lines are linear fits to the irradiance residuals in the two 'precision solar pointed' periods. 1980 residuals are clustered above those from the 1984–86 period. Solid lines: regression predicted from the correlation between white-light faculae and Ca-plage.

in the average irradiance of each group occurs. The most pronounced effects are for 1980, due to the more frequent occurrence of sunspots in that period.

The third panels of Fig. 2 give the residuals after complete adjustment for active-region effects, both PSI and PFI. The average irradiance and the difference between the mean values of irradiance for both groups of results are decreased, but the tightness of the correlation perceptibly decreases relative to that in the previous panels. We interpret this additional scatter as error introduced by use of the Ca-plage areas as a proxy for true facular photometry.

It is most significant that our model's removal of the active region irradiance effects does not produce convergence of the two groups of results. There remain two distinct régimes of correlation between irradiance and solar activity index, for both R_z and F_{10} , corresponding to periods of maximum and minimum solar activity. This is a clear indication that an additional irradiance component with a solar cycle time-scale is required to explain the long term trend.

Another approach in our investigation of a solar cycle irradiance component is illustrated in Fig. 3, which shows the sunspot-adjusted total irradiance residuals (ACRIM I results + PFI) plotted against the modelled facular irradiance component for the two 'precision solar pointed' periods of SMM operation. The 1980 data and the 1984–86 data naturally separate into two domains. The slopes of linear least-mean-square fits to both groups of data (dashed lines) are nearly the same, and agree quite well in slope with the PFI model predictions (solid lines). As with the correlations with R_z and F_{10} , the two distinct régimes of internally consistent results point to an additional source of irradiance variation linked to the solar cycle.

The additional component of solar luminosity variation revealed in Fig. 3 could be the white-light counterpart of the Lean *et al.*¹² 'third component' introduced to explain the solar ultraviolet irradiance variations. Foukal and Lean¹³ suggested this explanation using the solar full-disk 10,830 Å He-emission excess as a means of observing the 'active network', the name of this additional component.

Other explanations for the apparent solar cycle luminosity-dependence exist. One of particular interest is a pulsation of the Sun involving global thermal and transport properties on the magnetic time scale.

The energy involved in the solar cycle component cannot be accurately estimated without more precise calibration of the PFI index, or direct facular photometry, but the time series in Fig. 1 demonstrates that its magnitude rivals that of the individual large sunspot groups at their time of peak area. It is thus an appreciable fraction of 0.1% of the solar bolometric luminosity, and its behaviour during the forthcoming solar maximum will be of great interest for both solar physics and climatology.

The SMM/ACRIM I experiment is supported by NASA at the Jet Propulsion Laboratory. Work at UCSD was supported by a grant from NASA.

Received 4 November 1987; accepted 28 January 1988.

- Willson, R. C. *Space Sci. Rev.* **38**, 203–242 (1984).
- Willson, R. C., Hudson, H. S., Fröhlich, C. & Brusa, R. W. *Science* **234**, 1114–1117 (1986).
- Eddy, J. A. *Climate Change* **1**, 173–190 (1977).
- J. R. Hickey in *Advances in Absolute Radiometry* (ed. Foukal, P. V.) 30–33 (AER, Cambridge, MA, 1985).
- Willson, R. C., Gulkis, S., Janssen, M., Hudson, H. S. & Chapman, G. A. *Science* **211**, 700–702 (1981).
- Willson, R. C. *J. geophys. Res.* **86**, 4319–4326 (1982).
- Oster, L. F., Schatten, K. H. & Sofia, S. *Astrophys. J.* **256**, 768–772 (1982).
- Sofia, S., Oster, L. & Schatten, K. *Solar Phys.* **80**, 87–89 (1982).
- Chapman, G. A. in *Solar Irradiance Variations on Active Region Time Scales* (eds La Bonte, Chapman, Hudson & Willson) 73–89 (NASA, Washington, 1983).
- Hirayama, T., Okamoto, T. & Hudson, H. S. *ibid* 43–58.
- Hudson, H. S. & Willson, R. C. in *The Physics of Sunspots* (eds Cram, L. & Thomas, J.) 434–435 (Sacramento Peak Observatory, New Mexico, 1981).
- Lean, J. L. *et al. J. Geophys. Res.* **87**, 10307–10317 (1982).
- Foukal, P. & Lean, J. *Astrophys. J.* (in the press).

Methane concentration in the glacial atmosphere was only half that of the preindustrial Holocene

B. Stauffer, E. Lochbrunner, H. Oeschger
& J. Schwander

Physics Institute, University of Bern, 3012 Bern, Switzerland

Air entrapped in bubbles of cold ice has essentially the same composition as that of the atmosphere at the time of bubble formation. Measurements on ice core samples from Byrd Station (Antarctica) and Dye 3 (Greenland) show that the atmospheric methane concentration was only about 350 parts per 10^9 by volume (p.p.b.v.) during the last glaciation, compared with a mean pre-industrial level of about 650 p.p.b.v. and a present value of 1,650 p.p.b.v.

Methane is an atmospheric radiatively active trace constituent. After CO_2 it is second in importance as a 'greenhouse' gas. Additionally, changes in the atmospheric mixing ratio have important implications for the chemistry of the troposphere and stratosphere. The atmospheric concentration of methane has been recorded continuously since 1977. The concentration in 1951 can be deduced from infrared solar spectra, measured at that time. To investigate earlier concentrations, it is possible to analyse air from bubbles of ice samples from cold polar ice sheets.

Today's atmospheric methane mixing ratio is 1,650 p.p.b.v. (ref. 1) and was 1,140 p.p.b.v. in 1951². It is increasing at present at a rate of 1–2% per year³. According to measurements on ice samples, the concentration was stable at a level of 650 p.p.b.v. for several millenia before the obviously anthropogenically induced increase started about 200 yr ago. The record of the past 3,000 yr is well established, since it is based on numerous measurements on different ice cores in different laboratories^{4–6}. For earlier epochs only a few sporadic results are available. In this paper we discuss the record of the atmospheric methane concentration during the last glaciation and during the transition

Table 1 Methane concentration (in p.p.b.v.) in air extracted from ice samples from Dye 3 and Byrd Station

Depth (m)	Age of air (yr BP)	Methane concentration (p.p.b.v.)
<i>Dye 3</i>		
1185.03	3,400	627 ± 25
1670.91	7,700	663 ± 30
1675.26	7,800	680 ± 27
1681.40	7,900	645 ± 37
1789.00	10,800	477 ± 25
1808.39	14,000	650 ± 32
1818.90	16,000	389 ± 32
1828.90	19,000	327 ± 30
1836.81	21,000	358 ± 41
1896.83	39,800	561 ± 31
1898.33	40,500	411 ± 32
1899.33	41,000	427 ± 33
1951.77	73,000	468 ± 25
1990.22	100,000	468 ± 25
<i>Byrd Station</i>		
147.22	550	667 ± 25
153.82	600	647 ± 25
750.04	7,100	615 ± 25
999.63	10,600	638 ± 25
1200.68	14,300	460 ± 25
1395.97	22,400	341 ± 27
1501.30	27,900	360 ± 25
1601.13	33,500	385 ± 25
1795.76	46,600	457 ± 27
1853.36	51,100	505 ± 31

from the glacial to the postglacial epoch, based on measurements on two ice cores, one from Antarctica and one from Greenland.

The Antarctic core was drilled in 1967–68 by the US Cold Regions Research and Engineering Laboratory at Byrd Station (79°59' S, 120°01' W) in West Antarctica⁷. The total length of the core is 2,184 m. The mean annual air temperature at Byrd is –28 °C, and the annual accumulation 160 kg m⁻². No melt-layers are observed in the Byrd core. The Greenland core was drilled at Dye 3 (65°11' N, 43°50' W) during the summer months 1979–1981 as part of an international collaboration (Greenland Ice Sheet Program) between the United States, Denmark and Switzerland⁸. The drilling reached bedrock at 2,037 m depth. The mean annual air temperature at Dye 3 is –19.6 °C, and the annual accumulation 500 kg m⁻². During summer, melting and refreezing of a thin surface layer occurs regularly. The melt-layers, and to a certain extent the ice between the meltlayers, are enriched in highly soluble air components like CO₂. The influence on the methane concentration, however, seems to be negligible, as comparisons with results from the ice cores from Byrd Station and Dye 3 show.

There are principally two different methods of extracting air from ice samples. The bubbles are either opened mechanically at low temperature (dry extraction method), or the air is released by melting the ice sample (melt extraction method). We showed that both methods give, within the limits of error, the same results⁸. For the measurements which are discussed in this paper, we applied only the melt extraction method since it is easier to handle and the contamination is smaller and better reproducible.

For the extraction, a sample of 500–800 g ice is placed in a glass cylinder. The cylinder with the sample is evacuated for 20 min to further clean the sample surface. Subsequently the ice sample is melted in 30–40 min. The air released is dried and collected continuously by condensation at a temperature of 15–20 K. The air collected is analysed with a gas chromatograph (Hewlett-Packard 5880A) equipped with a flame ionization detector. As standard gas a mixture of N₂, O₂, Ar and CO₂ in

atmospheric composition and 980 ± 30 p.p.b.v. CH₄ (from Messer Griessheim) is used. Each air sample is measured five times. Sample and standard gases are measured alternately. The contamination caused by the extraction procedure is determined by analysing ice containing no air bubbles and no methane. Before melting a sample of artificially grown, very pure single-crystal ice, a small amount of standard gas is added to the glass cylinder. The tests show a contamination with CH₄ corresponding to a rise of the CH₄ concentration by 40 ± 10 p.p.b.v. for a sample size of 500 g ice. All results have been corrected accordingly. The precision of our analyses, including extraction, is within ± 25 p.p.b.v.; the accuracy is further limited by the standard gas, which gives an additional uncertainty of 3% in the methane concentration.

We measured 10 samples from Byrd Station and 14 samples from Dye 3. The results are given in Table 1 and presented in Fig. 1. In the figure the results are plotted as a function of the estimated age. The ages for Byrd Station are estimated according to Hammer and Clausen⁹. Their dating relies on a continuous measurement of the acidity along the whole core. The ages of the Dye 3 core are the ones given by Dansgaard *et al.*¹⁰. The difference between the age of the ice and the age of the enclosed bubbles has been taken into account. The results from both cores show, in good agreement, a decrease in the CH₄ concentration from 500 p.p.b.v. 60,000 yr BP to 350 p.p.b.v. about 20,000 yr BP. The decrease has been followed by a rapid increase to the Holocene value of 650 p.p.b.v.

The question might arise whether a lower CH₄ concentration could be due to a depletion of methane by reactions with impurities in the ice. The concentration of dust and soluble impurities is indeed higher in ice from the last glaciation. There is, however, a difference in the insoluble and soluble impurity concentration between the glacial part of the Byrd core and the Dye 3 core of more than an order of magnitude^{11,12}. Based on the good agreement between the methane concentrations measured in the two cores, including the part representing the glacial epoch, such an effect can be excluded. We conclude, therefore, that the results reflect a shift of the atmospheric methane concentration from about 350 p.p.b.v. during the late part of the glacial period to about 650 p.p.b.v. in the Holocene.

Previously, only one value of the atmospheric CH₄ concentration for the glacial epoch has been published⁴. The concentration of 650 p.p.b.v., based on the analysis of an ice sample from Dye 3 from 1,950.7 m depth, is significantly higher than our result for a sample only 1 m deeper (corresponding to an age difference

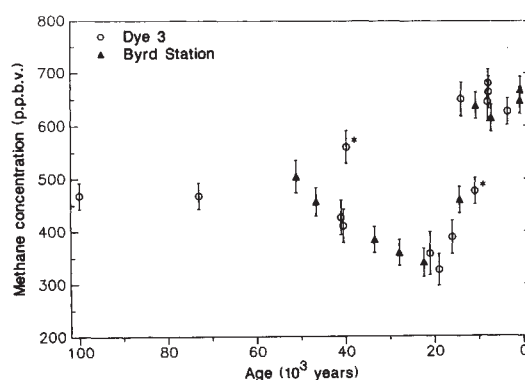


Fig. 1 Measured CH₄ concentration plotted against estimated ages of air enclosed in bubbles of ice. Circles represent results from the ice core from Dye 3. Triangles represent results from the ice core from Byrd Station. The error bars include the uncertainties in the gas extraction and the gas chromatographic analysis. An additional shift in either direction of up to 3% is possible due to the calibration of the standard gas. The values marked with an asterisk deviate from the general course and are discussed separately in the text.

of about 500 yr). Further measurements are needed to show whether or not this difference reflects a real fluctuation.

The increase in the atmospheric CH_4 concentration at the end of the last glaciation is due to either an increase in methane sources or a decrease in methane sinks, or both. The main sink of atmospheric methane is its reaction with OH. It is very difficult to estimate the OH concentration during the last glaciation. We assume that the lifetime of CH_4 was the same as today, and that changes in the methane concentration are rather caused by changes of the sources. The global source of methane at present is estimated by Rasmussen & Khalil³ to be 553 Tg yr^{-1} corresponding to a mean atmospheric lifetime of methane of 8 yr. The natural wetlands contribute at present 150 Tg yr^{-1} . An atmospheric mixing ratio of 650 p.p.b.v. would correspond, assuming a constant lifetime, to a source of 220 Tg yr^{-1} . If we assume that the contribution by natural wetlands was about constant at 150 Tg yr^{-1} during the whole Holocene, the wetlands would have been the dominant source until about 200 yr ago. It seems quite possible that the methane production by this major source was reduced during the glacial epoch because the area of the wetlands was smaller and their temperature was lower.

The increase of the atmospheric CH_4 concentration from 350 to 650 p.p.b.v. also had climatic implications. The climatic forcing was certainly much smaller than that, caused by the increase of the CO_2 concentration from about 200 p.p.m.v. to 280 p.p.m.v.¹³ We estimate, based on calculations by Tricot and Berger¹⁴ which include some feedback effects, a global temperature increase of about 0.1°C caused by the CH_4 increase. For this estimate we suppose, however, that the sensitivity of the global climate was the same at that time as it is today.

There are two values in the Dye 3 data set which deviate from the smooth course of our record. The low value at 1,789 m depth falls into the Younger Dryas cold period for which we also found lower CO_2 concentrations in the same core. The relatively high value at a depth of 1,896.83 m corresponds to a rather warm period during the last glaciation, for which we observed in the Dye 3 core also high CO_2 concentrations¹⁴. The CH_4 deviations found in the Dye 3 core which go parallel with deviations of the CO_2 concentration in the same core, but are not yet found in the Byrd core, will be discussed later, when more data are available. These deviations are interesting in respect of possible short-term fluctuations. They do not affect our conclusion concerning the long-term record, i.e. that the atmospheric CH_4 concentration was only about 350 p.p.b.v. during the late part of the last glaciation and increased to 650 p.p.b.v. at the end of the glacial epoch.

The samples from Byrd Station were provided with the permission of the Department of Polar Programs of US NSF by C. C. Langway. The fieldwork in Greenland and the laboratory analyses were supported by the Swiss NSF.

Superconductivity near 30 K without copper: the $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_3$ perovskite

R. J. Cava, B. Batlogg, J. J. Krajewski, R. Farrow, L. W. Rupp Jr, A. E. White, K. Short, W. F. Peck & T. Kometani

AT&T Bell Laboratories, Murray Hill, New Jersey 07974, USA

It is well known that the breakthrough of Bednorz & Müller¹ in discovering superconductivity in $(\text{La}, \text{Ba})_2\text{CuO}_4$ was inspired in part by their knowledge of the superconducting properties of $\text{Ba}(\text{Pb}, \text{Bi})\text{O}_3$ (ref. 2). With a transition temperature, T_c , of $\sim 12 \text{ K}$, that compound was not generally considered anomalous despite the fact that its T_c is 3–5 times higher than that of traditional superconductors with comparable density of states^{3–5}. The increases in T_c for copper-oxide-based materials continue to generate worldwide excitement, but from both a chemical and theoretical point of view it would also be exciting if high- T_c superconductivity were observed in another class of materials. Here we report the results of experiments leading us to the single-phase perovskite $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_3$, which has a magnetically determined onset temperature of 29.8 K —a T_c considerably higher than that of conventional superconductors and surpassed only by copper-containing compounds. Superconductivity in this compound occurs within the framework of a three dimensionally connected bismuth–oxygen array. These results suggest that further research toward exploring the limiting T_c s for bismuth-oxide-based, high-temperature superconductors might be fruitful.

$\text{Ba}(\text{Pb}, \text{Bi})\text{O}_3$ is made superconducting by mixing of atoms on the electronically active perovskite 'B' sites. Alternatively, 'A'-site doping, in analogy to $(\text{La}, \text{Ba})_2\text{CuO}_4$, can be effective in inducing superconductivity: for samples which had melted at some time during their synthesis, Mattheiss *et al.*⁶ indeed observed 4% superconductivity in multi-phase samples of $(\text{Ba}, \text{M})\text{BiO}_x$ ($\text{M} = \text{K}, \text{Rb}$), with magnetically determined onset temperatures of 22 and 15 K, respectively. In early studies of the $\text{Ba}-\text{K}-\text{Bi}-\text{O}$ phase diagram under conditions which avoided melting, we found no bulk superconducting compound. In the following, we describe a reproducible alternative synthetic route which results in bulk perovskite-structure superconductors with T_c s near 30 K. Preliminary structural, chemical and magnetic properties are also reported.

Owing to the expected volatility of alkali oxides, we decided to investigate the $\text{Ba}-\text{K}-\text{Bi}-\text{O}$ phase diagram using sealed silver tubes. Starting materials were BaO , KO_2 , and Bi_2O_3 , and the reactions were carried out at 675°C for 3 days. Materials pro-

Table 1 Reaction conditions and superconducting properties of $\text{Ba}(\text{K}, \text{Rb})\text{-Bi-O}$ superconductors

Starting mixture	Reaction temperature ($^\circ\text{C}$)	Reaction time	Oxygen anneal temperature ($^\circ\text{C}$)	T_c (K)	Diamagnetism (%)
$\text{Ba}_{0.7}\text{K}_{0.45}\text{BiO}_x$	675	3 days	475	29.2	20
$\text{Ba}_{0.7}\text{K}_{0.6}\text{BiO}_x$	675	3 days	475	29.8	25
$\text{Ba}_{0.65}\text{K}_{0.52}\text{BiO}_x$	675	3 days	425	28.3	20
$\text{Ba}_{0.65}\text{K}_{0.7}\text{BiO}_x$	675	3 days	475	29.2	23
$\text{Ba}_{0.6}\text{K}_{0.8}\text{BiO}_x$	675	3 days	425	28.0	20
			475	29.8	30
$\text{Ba}_{0.5}\text{K}_{0.75}\text{BiO}_x$	825	2.5 h	425	24.7	25
			475	26.5	24
$\text{Ba}_{0.6}\text{Rb}_{0.6}\text{BiO}_x$	675	3 days	425	28.6	3
$\text{Ba}_{0.5}\text{Rb}_{0.75}\text{BiO}_x$	675	3 days	425	28.5	5
$\text{Ba}_{0.5}\text{Rb}_{0.75}\text{BiO}_x$	825	2.5 h	425	25.5	6
			475	25–26	7

Received 25 January; accepted 4 March 1988.

- Ramanathan, V., Cicerone, R. J., Singh, H. B. & Kiehl, J. T. *J. geophys. Res.* **90**, 5547–5566 (1985).
- Rinsland, C. P., Levine, J. S. & Miles, T. *Nature* **318**, 245–249 (1985).
- Khalil, M. A. K. & Rasmussen, R. A. *J. geophys. Res.* **88**, 5131–5144 (1983).
- Craig, H., & Chou, C. C. *Geophys. Res. Lett.* **9**, 1221–1224 (1982).
- Rasmussen, R. A. & Khalil, M. A. K. *J. Geophys. Res.* **89**, 11,599–11,605 (1984).
- Stauffer, B., Fischer, G., Neftel, A. & Oeschger, H. *Science* **299**, 1386–1388 (1985).
- Ueda, H. T. & Garfield, D. E. *Techn. Report No. 231* (US Cold Regions Research and Engineering Laboratory, Hanover, New Hampshire, 1969).
- Langway, C. C., Oeschger, H. & Dansgaard, W. *Geophys. Monogr.* **33**, (AGU Washington, DC, 1985).
- Hammer, C. U. & Clausen, H. B. *Ann. Glaciol.* **7**, 214 (1985).
- Dansgaard, W., Clausen, H. B., Gundestrup, N., Hammer, C. U., Johnsen, S. F., Kristinsdottir, P. M. & Reeh, N. *Science* **218**, 1273–1277 (1982).
- Cragin, J. H., Herron, M. M., Langway, C. C. & Klouda, G. A. in *Polar Oceans* (ed. Dunbar, M. J.) 617–631 (Calgary, 1977).
- Hammer, C. U., Clausen, H. B. & Dansgaard, W. *Geophys. Monogr.* **33**, 90–94 (AGU Washington, DC, 1985).
- Neftel, A., Moor, E., Oeschger, H. & Stauffer, B. *Nature* **315**, 45–47 (1985).
- Tricot, Ch. & Berger, A. *Clim. Dyn.* **2**, 39–61 (1987).
- Stauffer, B., Hofer, H., Oeschger, H., Schwander, J. & Siegenthaler, U. *Ann. Glaciol.* **5**, 160–164 (1984).

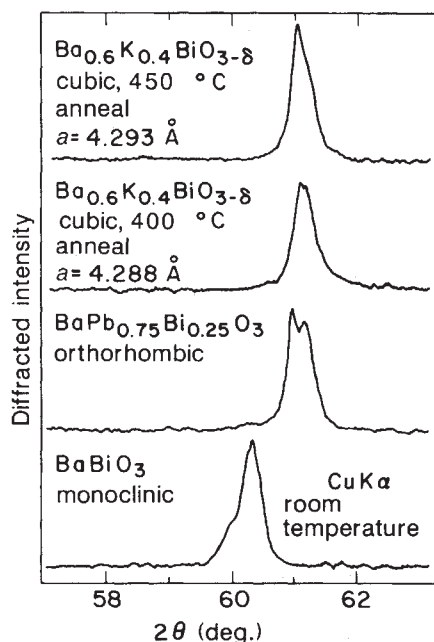


Fig. 1 Greatly expanded powder diffraction patterns near the 220 reflection for $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$ annealed at 475 and 425 °C in oxygen, and for $\text{BaPb}_{0.75}\text{Bi}_{0.25}\text{O}_3$ and BaBiO_3 , all measured on the same diffractometer.

duced by this technique allowed us to identify the perovskite-structure compound as the bulk superconductor in this chemical system. When the starting materials are mixed in the stoichiometric perovskite ratios, $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$, only a few per cent superconductivity is observed, even in materials which appear to be single phase by X-ray diffraction. The amount of superconductivity observed increases dramatically when excess alkali is employed in the starting mixture. Further, the as-synthesized materials are not superconducting, but require a low-temperature anneal in oxygen to optimize the superconducting properties. Exploring this multi-dimensional parameter field led us to the following preliminary optimal conditions: single-phase perovskite of stoichiometry $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$ is prepared by adding 100% excess K_2O , in 2-g batches, heated at 675 °C for 3 days, in 0.25-in. diameter sealed silver tubes, 6 in long, and sealed in turn in evacuated quartz tubes. The material removed from the tubes is dark red or brown in colour. It is ground and then annealed in flowing oxygen at 475 °C for 45 min, and quickly (<5 min) cooled in the oxygen flow. The resulting powder has a deep blue-black colour. It is then pressed into pellets for measurement. Oxygen anneals at higher temperatures or longer times result in the loss of potassium from the $(\text{Ba},\text{K})\text{BiO}_{3-\delta}$ compound. Any excess K oxide present is not visible in an X-ray diffraction pattern down to a detectability limit of 1% of the main perovskite peak, but is clearly present as a transparent white coating on the $(\text{Ba},\text{K})\text{BiO}_{3-\delta}$ grains when initially removed from the silver tubes. This material is almost fully volatilized away during the oxygen anneal. The overall chemical composition of the optimal sample was determined by atomic emission analysis to be $\text{Ba}_{0.6}\text{K}_{0.54}\text{BiO}_x$, thus suggesting the presence of a small potassium excess in a form not detectable by X-ray diffraction. Because of this the measurement of the oxygen concentration was considered unreliable. We expect it, however, to be close to the ideal value ($\delta \approx 0$). Some alternative synthetic conditions, and the resulting superconducting properties are summarized in Table 1.

X-ray powder diffraction was used to determine the phase purity of the reaction products. All samples have predominantly the perovskite structure, with a maximum of 10% impurity, under non-ideal synthesis conditions, identified as unreacted Bi_2O_3 . But single-phase perovskites (to ~1% detectability limit)

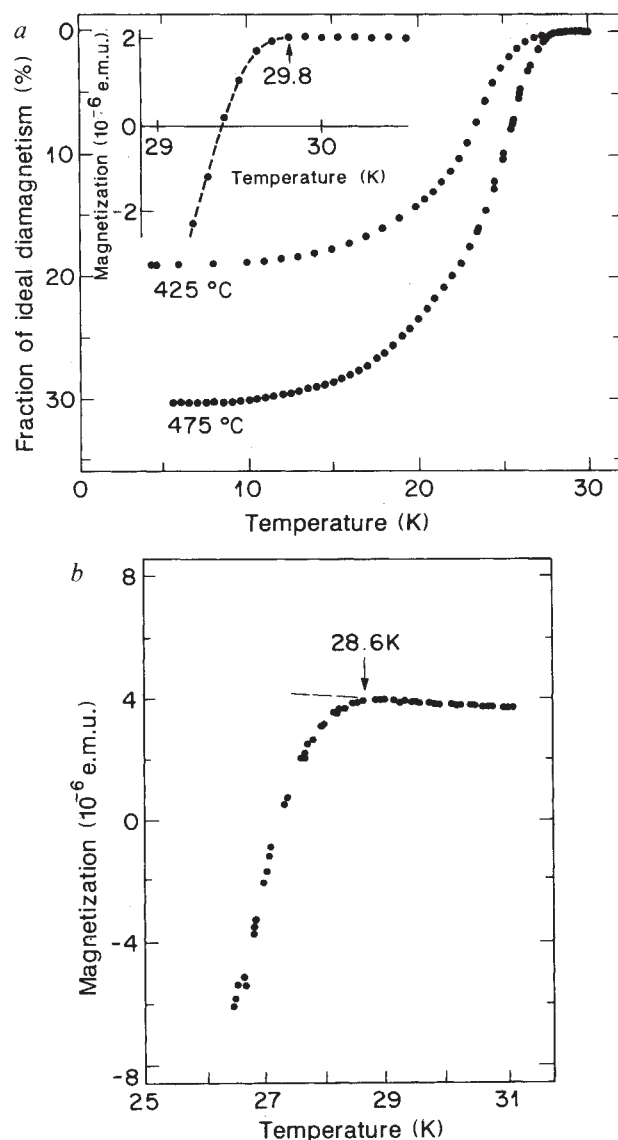


Fig. 2 *a*, Temperature-dependent magnetization for $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$ samples cooled in a field of 19 Oe. Inset shows region near T_c for the sample annealed at 475 °C in oxygen. *b*, Temperature-dependent magnetization for multi-phase $\text{Ba}_{0.6}\text{Rb}_{0.4}\text{BiO}_x$ cooled in a field of 19 Oe in the vicinity of T_c .

can be prepared in a reproducible manner for $\text{Ba}_{1-x}\text{K}_x\text{BiO}_{3-\delta}$ for $0 < x < 0.5$. From these materials we found the highest T_c and largest superconducting volume fraction for $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$.

The powder diffraction pattern of the 'optimal' compound is indicative of single-phase cubic perovskite, determined by least-squares fits to the measured positions of six reflections to $2\theta = 65^\circ$ ($\text{CuK}\alpha$). Figure 1 shows, on a greatly expanded scale, the 220 reflections from $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$, as annealed in oxygen at 425 and 475 °C, compared with those of BaBiO_3 and $\text{BaPb}_{0.75}\text{Bi}_{0.25}\text{O}_3$. The lattice parameters of the current compounds are significantly smaller than that of BaBiO_3 , reflecting the decrease in the size of Bi due to its increased formal oxidation. The lattice parameters increase on going from the 425 °C to the 475 °C anneal ($a_0 = 4.2881(5)$ Å and $a_0 = 4.2926(7)$ Å, respectively) due to an increased uptake of oxygen at the higher temperature. We show the corresponding reflection of $\text{BaPb}_{0.75}\text{Bi}_{0.25}\text{O}_3$ to indicate that the resolution of this diffractometer would be sufficient to detect small departures from cubic symmetry such as are present in that compound. No superlattice reflections, characteristic of simple cell doubling, are observable

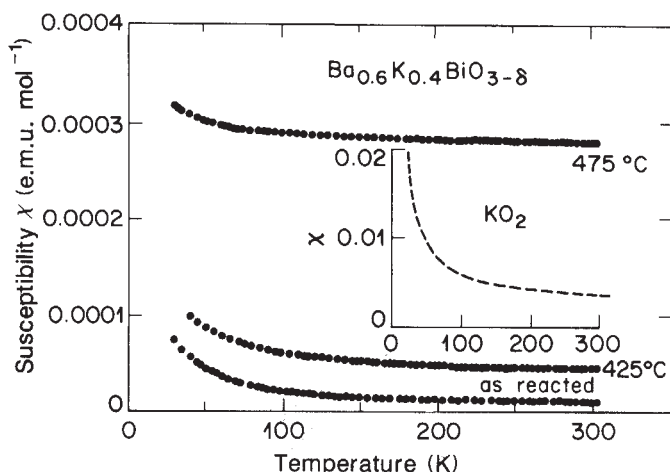


Fig. 3 Normal-state susceptibility for $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$ in the as-prepared (675 °C) material, and the material annealed in oxygen at 425 and 475 °C.

in $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$ at intensities above the detectability limit. Further studies, involving neutron diffraction, are necessary to clarify the details of the crystal structure, and its temperature dependence.

As the pressed powders are not suitable for quantitative transport measurements, superconductivity was studied magnetically using SQUID magnetometers. Figure 2a shows the signal for samples cooled in an external field of 19 Oe. The scale refers to the diamagnetism expected for an ideal diamagnet with the same solid volume. Macroscopic or microscopic demagnetization corrections have not been applied. The samples are porous and the real fraction of superconducting volume is higher than the measured one. The sample oxidized at 475 °C has not only a larger superconducting volume fraction, but also has a higher T_c . As shown in the inset, the magnetization deviates from its normal-state value at a temperature of 29.8 K, indicating the onset of superconductivity, and becomes negative at 29.4 K. Onsets above 29 K are observed for a variety of samples, in two different magnetometers. Rubidium-substituted samples, prepared in a similar manner employing Rb_2O in place of K_2O , exhibit a smaller volume fraction of superconductivity, yet have similar onset temperatures (Table 1). Representative data are presented in Fig. 2b. Owing to the small superconducting volume fraction and the poorer phase purity of these materials we cannot unambiguously identify the origin of the superconductivity in the Ba-Rb-Bi-O chemical system, however it would be surprising if it were not the perovskite.

In a first attempt to elucidate the electronic properties of the $\text{Ba}_{0.6}\text{K}_{0.4}\text{BiO}_{3-\delta}$ superconductor, we have measured the normal-state susceptibility for the compound at three different stages in its synthesis. The data are presented in Fig. 3. The as-reacted samples (675 °C) are weakly paramagnetic (susceptibility, $\chi(300 \text{ K}) \approx 1 \times 10^{-5} \text{ e.m.u. mol}^{-1}$) with an additional Curie-Weiss-like contribution. We suggest that this contribution is due to the presence of $\sim 0.1\%$ of unreacted KO_2 which is highly magnetic (see, for example, ref. 8) with an effective magnetic moment, μ_{eff} , of $1.5\mu_B$ per formula unit, as we have directly measured on our starting material (see inset). Post-annealing in an oxygen atmosphere induces superconductivity, accompanied by a pronounced increase of normal-state paramagnetism. Again, we attribute the small low-temperature upturn in these curves to the presence of KO_2 , the amount of which decreases for the high-temperature treatment. The value of the normal-state susceptibility ($\sim 3 \times 10^{-4} \text{ e.m.u. mol}^{-1}$) for the best superconducting sample is comparable to that for $\text{YBa}_2\text{Cu}_3\text{O}_7$. An interpretation of its origin and its association with the superconducting compound seems premature, however, we point out that the normal-state susceptibilities of BaBiO_3 and $\text{BaPb}_{0.75}\text{Bi}_{0.25}\text{O}_3$

are negative, and approximately equal to the estimated core diamagnetism (-6 and $-9 \times 10^{-5} \text{ e.m.u. mol}^{-1}$ respectively). Although the increased paramagnetism, on going from the non-superconducting to the superconducting stage in the present compound, is what would be expected on increasing the free carrier concentration, the opposite was in fact observed for $\text{Ba}(\text{Pb}, \text{Bi})\text{O}_3$ (ref. 7). Thus, the magnetic properties of the bismuthate compounds are not well understood, and require further study before they can be used to draw conclusions concerning the electronic state of these materials.

We thank D. W. Johnson Jr for helpful discussions and for providing one of his samples, and J. P. Remeika and A. S. Cooper for crystals of BaBiO_3 and $\text{BaPb}_{0.75}\text{Bi}_{0.25}\text{O}_3$.

Received 5 April; accepted 15 April 1988.

1. Bednorz, J. G. & Müller, K. A. *Z. Phys.* **B64**, 189-193 (1986).
2. Sleight, A. W., Gillson, J. L. & Bierstedt, P. E. *Solid St. Commun.* **17**, 27 (1975).
3. Batlogg, B. *Physica* **126B**, 275 (1984).
4. Mattheiss, L. F., *Jap. J. appl. Phys.* **24**, suppl. 24-2, 6 (1985).
5. Kitazawa, K., Uchida, S. & Tanaka, S. *Physica* **135B**, 505-510 (1985).
6. Mattheiss, L. F., Gyorgy, E. M. & Johnson, D. W. *Jr Phys. Rev.* **B37**, 3745-3746 (1988).
7. Batlogg, B. *et al. Superconductivity in d- and f-band metals 1982* (eds Buckel, W. & Weber, W.) 401-403 (KFZ Karlsruhe).
8. Zumsteg, A., Ziegler, A., Känzig, W. & Bösch, M. *Phys. Cond. Matter* **17**, 267 (1974).

Superconductivity in $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ in a 100 tesla magnetic field

Koichi Nakao*, Noboru Miura*, Kiyoshi Tatsuhara*, Shin-ichi Uchida†, Hidenori Takagi†, Takahiro Wada†‡ & Shoji Tanaka†

*Institute for Solid State Physics, The University of Tokyo, Roppongi, Mitato-ku, Tokyo 106, Japan

†Department of Applied Physics, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

The upper critical field (H_{c2}) of the high- T_c superconductor $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ (YBCO) is expected to be $>100 \text{ T}$ at 0 K^{1-6} . Because magnetic fields of $>100 \text{ T}$ can be produced only for a short time and by destructive methods, direct measurements of H_{c2} at these field strengths have not so far been performed. Here we report the first observation of superconductivity at $>100 \text{ T}$, using a method in which a voltage induced by a magnetization jump is measured in pulsed high magnetic fields. Superconductivity in YBCO was confirmed up to 101 T at 6 K .

At relatively low fields, H_{c2} is usually determined resistively or magnetically at various temperatures. Several measurements of H_{c2} in YBCO have been made at fields up to 60 T , produced by conventional pulsed magnets^{1,2,5,6}. To obtain fields of $>100 \text{ T}$, one must use destructive methods, and the experimental situation is quite different. The measurement of the electrical resistivity is difficult, because the rise time of the magnetic field is very short (typically several microseconds), and a very large voltage is induced in a small loop of lead wire ($\sim 100 \text{ V}$ in a loop of 1 mm^2 area). The measurement of the magnetization is also very difficult because the magnetization due to the superconducting current ($M \approx 10^{-2} \text{ T}$) is much smaller than the applied field ($\sim 10^2 \text{ T}$), and a very large spurious voltage, which is inevitable in destructive methods, is induced in a pick-up coil.

The magnetization of YBCO is negative when the external field is increasing and positive when the field is decreasing. The magnetization curve is very similar to that predicted by the critical-state model⁷⁻⁸. The polarity of the magnetization changes abruptly just after the external field reaches its maximum value

‡ Permanent address: Central Research Laboratory, Matsushita Electric Industrial Co. Ltd, Moriguchi, Osaka 570, Japan.

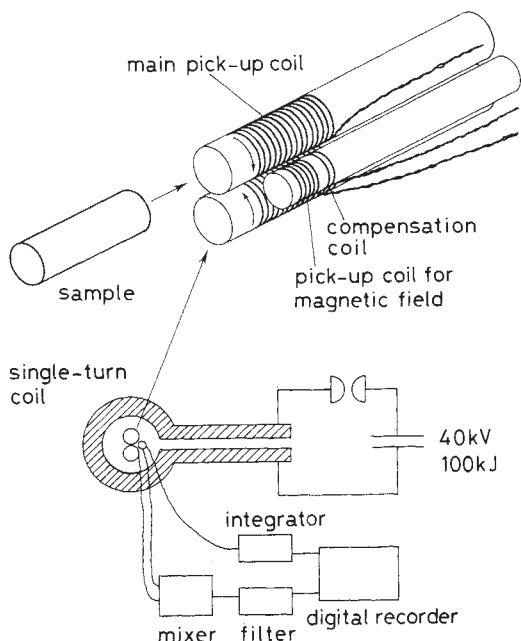


Fig. 1 Diagram of the measuring system.

and starts to decrease. In an experiment in pulsed fields, this change of polarity of the magnetization occurs very rapidly and a large voltage is induced in a pick-up coil wound around the sample, which detects the derivative of the magnetic flux. It is possible to observe this voltage even in an ultra-high and very rapidly changing magnetic field, although the measurement of the whole magnetization curve is very difficult owing to the reasons mentioned above. We consider this voltage to be direct evidence that the material is still superconducting at the maximum value of the pulsed field.

Ultra-high magnetic fields were produced by the single-turn coil method. In this method, a large current (2.5 MA) is supplied from a low-impedance capacitor bank to a single-turn coil, and we obtain magnetic fields of 150 T with a rise time of 2.5 μ s. The single-turn coil explodes after the generation of the field, owing to the large Maxwell stress, but samples are not destroyed because the coil explosion is directed outwards⁹.

Sample preparation and superconducting properties have been reported elsewhere¹⁰. The sample was powdered and embedded in phenyl salicylate and packed in a glass capillary, so that the particles were isolated from one another. The total weight of the powder was 24 mg. The particle size was estimated to be 2–6 μ m by inspection with a microscope. The sample was cooled to ~ 6 K in flowing liquid helium.

The design of the pick-up coil system is similar to that for the usual magnetization measurement in pulsed fields. The pick-up coil consists of alternately wound positive and negative 15-turn windings to cancel the induced voltage due to the external magnetic field. A better cancellation can be achieved by mixing in a part of the voltage induced in a two-turn compensation coil which is wound independently and located close to the main pick-up coil. The pick-up coil system and the single-turn coil are shown in Fig. 1. The sample is put into either the positive or the negative windings of the main pick-up coil. When the signal is small, we make two measurements under the same conditions except that the sample is put in the positive windings in one measurement and in the negative windings in the other. Subtracting one record from the other numerically allows us to improve the signal-to-noise ratio.

Figure 2a shows an example of the detected voltage signal. A sharp pulse indicated by an arrow represents the magnetization jump at the maximum of the external field. The signal that

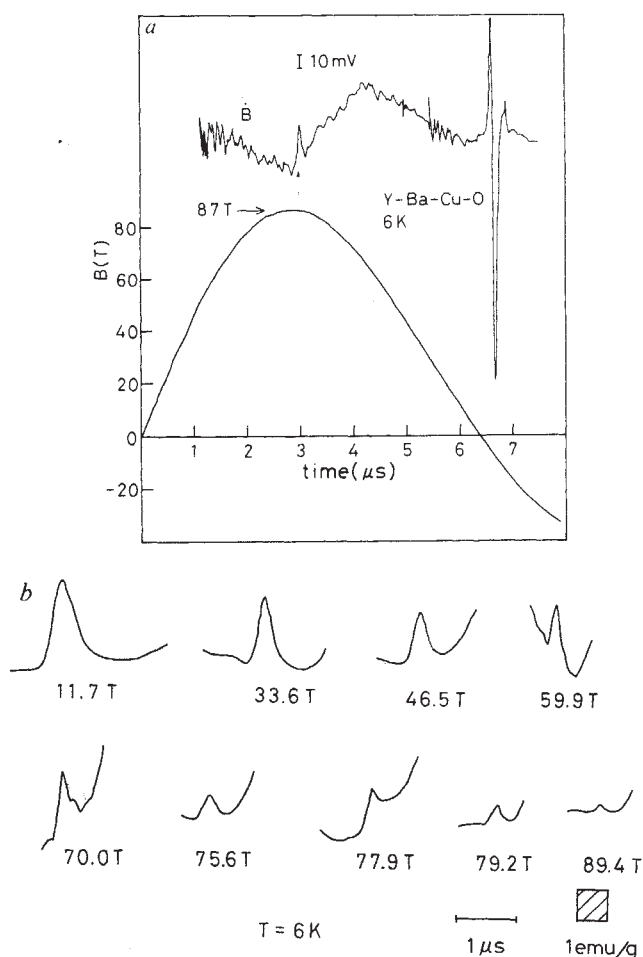


Fig. 2 *a*, An example of the traces for external magnetic field (lower trace) and induced voltage (upper trace) in the pick-up coil, obtained at 6 K with an YBCO sample. The voltage signal is proportional to the change in magnetization of the sample. *b*, Induced voltage at the peak of the external magnetic field for various maximum values of the field. The area under the curve is proportional to the sample magnetization; the area corresponding to 1 e.m.u. g^{-1} is shown.

is seen when the external field goes through zero corresponds to a large diamagnetism in the low-field region. This result shows unambiguously that the material is superconducting at 87 T at 6 K. Figure 2b shows the voltage pulses measured at various values of the peak external field.

The integral of the voltage pulse is proportional to the magnetization jump at the maximum value of the external field. Values of the magnetization jump ΔM are plotted as a function of the magnetic field in Fig. 3. ΔM decreases monotonically as the magnetic field increases, and it seems to disappear just above 100 T. This does not necessarily mean that $H_{c2} \approx 100$ T at this temperature, because ΔM may become undetectably small, approaching zero at higher fields. Moreover, it is possible that ΔM decreases because of the large dB/dt . It can be concluded, however, that $H_{c2}(0\text{ K})$ is ≥ 100 T.

Concerning the effect of dB/dt , we compared the result obtained with the present technique (with rise time 2.5 μ s) with similar measurements in longer pulsed fields up to 25 T produced by a multi-turn solenoid with rise time 13 ms. All of the experimental points in Fig. 3 were obtained using the present technique. The magnitude of the hysteresis at 4.2 K in the long-pulse measurement was ~ 7 e.m.u. g^{-1} at 10 T and did not decrease substantially up to 20 T (ref. 7). The value obtained in the present experiment is 2–3 times smaller than this, implying that the magnetic hysteresis has sweep-speed dependence.

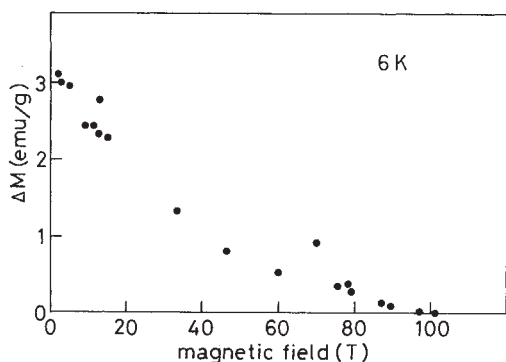


Fig. 3 Magnitude of magnetization jump as a function of the peak value of the external magnetic field.

Considering the slow relaxation of the magnetization that has been observed in measurements in steady fields⁷, we should expect a larger hysteresis in a faster magnetic-field pulse. In fact, we did observe such a tendency in measurements made on a powdered sample in longer pulsed fields; that is, the magnetic hysteresis at the same value of the magnetic field was slightly smaller when the peak field was smaller⁷. Note that the rise time of the pulsed magnetic field is nearly independent of the peak value, and dB/dt is proportional to the peak value. Thus, the dB/dt dependence of ΔM was opposite to the present results in higher fields with a faster rise time. On the other hand, in long-pulse measurements on bulk samples, the magnetic hysteresis was smaller when the peak field was larger⁷. This is probably due to the electric field induced by dB/dt , which is larger in larger samples. There are thus at least two competing factors which determine the dependence of M on the sweep speed of the magnetic field. At this moment, the effect of dB/dt is not yet completely clear.

The present experiments were performed on powdered samples, so the results represent an average over all particle orientations. In our method we measure the magnetization arising from the superconducting current in a plane perpendicular to the external magnetic field. In the critical-state model, the absolute value of the superconducting current density is assumed to be always equal to the critical current density. The crystal structure of YBCO is strongly two-dimensional, and the critical current density is greater in the a - b -plane than parallel to the c -axis¹¹; we therefore suppose that most of the magnetization comes from particles whose c -axis is nearly parallel to the external magnetic field. It is also well known that H_{c2} is lower when the magnetic field is applied along the c -axis (ref. 2)). Taking this into account, we cannot exclude the possibility that H_{c2} may be much higher when the magnetic field is applied perpendicular to the c -axis.

Within the approximation of the critical-state model, we can estimate the critical current density J_c from the magnitude of the magnetic hysteresis. In the case of a long circular cylinder with radius R , J_c is given by $J_c = 15\Delta M/R$ (ref. 12), where J_c is measured in $A\ cm^{-2}$, ΔM in gauss and R in cm. Although we cannot deduce an exact value of J_c in the present experiment because of the sweep-speed dependence of ΔM and the particle size distribution, we can still discuss the order of magnitude of J_c . Taking $2\ \mu m$ as an average value of R and $6.4\ g\ cm^{-3}$ as the density, we estimate J_c at 6 K to be $1.0 \times 10^6\ A\ cm^{-2}$ at 20 T and $0.5 \times 10^6\ A\ cm^{-2}$ at 60 T.

As discussed above, it is interesting to ask what will occur in a superconductor in a rapidly changing magnetic field, which gives rise to a high electric field within the sample. The present result shows that the sample remains superconducting not only in ultra-high magnetic fields but also in very rapidly pulsed fields, although the magnetic hysteresis is weakened by a factor of 2–3. Preliminary measurements on single crystals show that

the signal due to the magnetization jump can still be observed for single crystals whose size is 0.1–1.0 mm, although the electric field induced by the rapidly changing magnetic field is much larger than in the present experiment on the powdered sample.

Received 25 March; accepted 31 March 1988.

1. Nakao, K. *et al. Jap. J. appl. Phys.* **26**, 1187–1188 (1987).
2. Sakakibara, T. *et al. Jap. J. appl. Phys.* **26**, L1892–L1894 (1987).
3. Worthington, T. K., Gallagher, W. J. & Dinger, T. R. *Phys. Rev. Lett.* **59**, 1160–1163 (1987).
4. Oussena, M. *et al. Phys. Rev. B* **36**, 4014–4017 (1987).
5. Okuda, K., Noguchi, S., Yamagishi, A., Sugiyama, K. & Date, M. *Jap. J. appl. Phys.* **26**, L822–L824 (1987).
6. Orlando, T. P. *et al. Phys. Rev. B* **36**, 2394–2397 (1987).
7. Nakao, K. *et al. J. Phys. Soc. Japan* (submitted).
8. Gang Xiao *et al. Phys. Rev. B* **36**, 2382–2385 (1987).
9. Nakao, K. *et al. J. Phys.* **18**, 1018–1026 (1985).
10. Takagi, H. *et al. Jap. J. appl. Phys.* **26**, 1029–1030 (1987).
11. Dinger, T. R., Worthington, T. K., Gallagher, W. J. & Sandstrom, R. L. *Phys. Rev. Lett.* **58**, 2687–2690 (1987).
12. Bean, C. P., *Rev. mod. Phys.* **36**, 31–39 (1964).

Spatially resolved observation of the critical current in high- T_c superconducting films

R. Gross*, J. Bosch*, R. P. Huebener*,
J. Mannhart†, C. C. Tsuei†, M. Scheuermann†,
M. M. Oprysko† & C. C. Chi†

* Lehrstuhl Experimentalphysik II, Universität Tübingen,
D-7400 Tübingen, FRG

† IBM Research Division, T. J. Watson Research Center,
PO Box 218, Yorktown Heights, New York 10598-0218, USA

The superconducting properties of high-transition-temperature (high- T_c) copper-oxide superconductors depend sensitively on their microstructure. For example, the critical current density J_c of epitaxial $YBa_2Cu_3O_7$ films can be several orders of magnitude higher than that of fine-grained polycrystalline films and ceramics^{1,2}. Local variations in the size and distribution of features such as grains, grain boundaries, anisotropy and composition play an important role in determining the superconducting properties, and also profoundly affect the normal-state properties. A technique capable of probing superconducting properties with high spatial resolution—smaller than the grain size—is thus important not only for basic understanding, but also for device and microelectronics applications. Here we combine the techniques of micro-patterning^{3,4} and low-temperature scanning electron microscopy (LTSEM)^{5,6} to obtain the first spatially resolved observation of local values of the critical current I_c in high- T_c polycrystalline films. Both the range of values of I_c and the spatial distribution of the local I_c can be determined with a resolution approaching $1\ \mu m$. This new technique can be extended easily to the spatially resolved investigation of T_c distribution, flux pinning and flux flow⁶.

Experiments were performed with high- T_c films of Y-Ba-Cu-O, which were deposited at room temperature by d.c.-magnetron sputtering of two alloy targets (BaCu and YCu) on $SrTiO_3$ substrates³. The films were prepared in an Ar/O_2 atmosphere of $10\ \mu m$, with an oxygen partial pressure of $0.2\ \mu m$; annealing at 400 and 900 °C stabilized the films and formed the high- T_c phase³. The patterns were defined with an excimer laser micro-fabrication apparatus⁴. The pulsed laser (wavelength 193 nm, pulse duration 17 ns) illuminated an aperture which was projected with a reduction factor of 32 onto the samples. The samples were mounted on a stage which could be moved by computer-controlled stepper motors. Typically 10 pulses of ultraviolet light at a total fluence of $20\ J\ cm^{-2}$ were used to pattern the films. Typical lines of high- T_c material used in this study were $200\ \mu m$ long and $25\ \mu m$ wide.

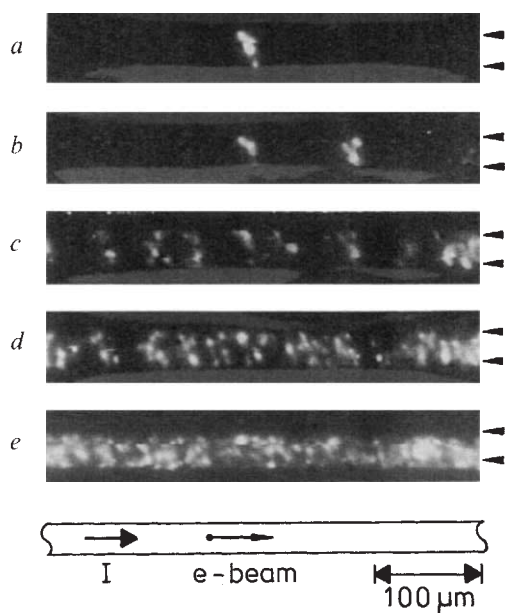


Fig. 1 Images obtained at 53.4 K with the following values of the bias current: *a*, 722 μA ; *b*, 912 μA ; *c*, 1.24 mA; *d*, 2.17 mA; *e*, 8.66 mA. The Y-Ba-Cu-O film extends horizontally beyond the field of view, as indicated schematically at the bottom. The arrows on the right of each image mark the location of the film boundaries.

The basic principle of LTSEM is to monitor an electrical response signal from a sample as a result of a local electron-beam heating. The specimen is scanned by the electron beam of a scanning electron microscope while it is kept at a constant temperature below T_c . The beam is modulated at some frequency f , causing a small local temperature modulation in the film at the coordinate point (x, y) of the beam focus and, hence, a small modulation of the local critical current value. During the scanning process the sample film is current-biased and the modulated voltage drop $\delta V(x, y)$ along the film is recorded as a function of the coordinate point (x, y) using a lock-in detector.

Since the width of our sample is only a few (~ 2 to 3) grains wide, we interpret $\delta V(x, y)$ as an indication of the critical current density at the local spot (x, y) being exceeded by the local bias current in the presence of local heating by the electron beam. The effect of current redistribution will probably drive the adjacent grains also into the normal state. If the bias current is increased in small steps and the sample is scanned by the electron beam after each step, the first signal $\delta V(x, y)$ appears at the coordinate point with the smallest I_c value. With increasing bias current I , regions with different local I_c values generate successively a signal $\delta V(x, y)$ when the bias current reaches the corresponding local I_c value. In this way the spatial distribution of the signal $\delta V(x, y)$ as a function of I maps the I_c distribution. Figure 1 shows a series of images obtained at 53.4 K at different values of the bias current. These data refer to a Y-Ba-Cu-O film 30 μm wide, 1.1 μm thick and 520 μm long (resistance at room temperature $\sim 300 \Omega$) with a resistive transition completed at $T_c = 82 \text{ K}$. The beam parameters were 1 nA, 25 kV, diameter $< 100 \text{ nm}$ and modulation frequency $f = 10 \text{ kHz}$. Bright regions indicate the locations where a beam-induced signal $\delta V(x, y)$ (typically 20 μV) has been generated during the scanning process; that is, where the bias current exceeds the local critical current I_c in the irradiated sample.

A series of $\delta V(x, y)$ pictures were taken for several bias currents I in monotonically increasing order. The first signal appeared at $I \approx 720 \mu\text{A}$. Figure 1*a* shows that the region with $I_c = 720 \mu\text{A}$ is localized at a distinct spot in the sample film, and that all other regions still remain superconducting. As the bias current was further increased, more and more resistive regions

appeared, as shown in Fig. 1*a-e*. At the highest bias current used (8.66 mA; Fig. 1*e*), some regions in the film, shown as the dark areas separating the bright regions, were apparently still superconducting. The results shown in Fig. 1 clearly demonstrate that along the direction of the current flow, the local I_c values in the film vary by more than a factor of ten. This result suggests that in this sample at 53.4 K, the local critical current density varies between 2,500 A cm^{-2} and 20,000 A cm^{-2} . We found that the observed patterns are highly reproducible and are not affected by thermal cycling. We note that the average size of the bright spots is about the grain size. Of course, the correlation between the variation in the local I_c values and corresponding variations in the microscopic structure of the film is a very important subject requiring further analysis of the microstructure of the specimen. We believe that this technique of LTSEM will be very useful to the study of anisotropy, percolation, grain boundaries and multi-phase problems in high- T_c films.

Partial financial support of this work by the Stiftung Volkswagenwerk is gratefully acknowledged. We thank J. Fischer, H.-G. Wener and J. Berosh for technical assistance.

Received 29 February; accepted 31 March 1988.

1. Chaudari, P., Koch, R. H., Laibowitz, R. B., McGuire, T. R. & Gambino, R. J. *Phys. Rev. Lett.* **58**, 2684-2686 (1987).
2. Oh, B. *et al. Appl. Phys. Lett.* **51**, 852-854 (1987).
3. Scheuermann, M. *et al. Appl. Phys. Lett.* **51**, 1951-1953 (1987).
4. Mannhart, J. *et al. Appl. Phys. Lett.* **52**, 1271 (1988).
5. Clem, J. R. & Huebener, R. P., *J. appl. Phys.* **51**, 2764-2773 (1980).
6. Huebener, R. P., Gross, R. & Bosch, J. *Z. Phys. B* (submitted).

Calculations of ^{29}Si MAS NMR chemical shift from silicate mineral structure

Barbara L. Sherriff & H. Douglas Grundy

Department of Geology, McMaster University,
1280 Main Street West, Hamilton, Ontario, Canada L8S 4M1

A simple correlation has been found between ^{29}Si magic-angle spinning nuclear magnetic resonance (MAS NMR) chemical shift and molecular geometry that is applicable to all silicate minerals. It is based on the magnetic anisotropy and valence of the bond between the oxygen and the second-neighbour cation to the silicon. The equations can be used to calculate the chemical shift and hence can be applied to assess the validity of structural models.

Since the first data were published on ^{29}Si MAS NMR of minerals^{1,2} there have been many attempts to correlate chemical shift with the structure of silicate minerals by using such geometrical parameters as Si-O-T (T being Si or Al) angle³⁻⁸, Si-O bond distance⁹⁻¹², number of bridging oxygens^{1,2,13}, type of next-nearest neighbours^{1,2,13-15} and type of cation in the formula^{8,11,13}. *Ab initio* calculations have also been used to explain ^{29}Si chemical shift¹⁶. Most of these correlations have been derived for specific types of silicate structure such as the silica polymorphs⁴ or zeolites^{2,7,14}. Notable exceptions are the initial correlations reported by Lippmaa and coworkers^{1,2} of chemical shift with degree of condensation of the silica tetrahedra and number of Al next-nearest neighbours and the correlations reported by Radeaglia and Engelhardt⁶ and Janes and Oldfield⁸ of the chemical shifts with Si-O-T angles.

In our study of the scapolite series of minerals¹⁵ we were able to modify the formula of Janes and Oldfield⁸ to resolve the complex ^{29}Si MAS NMR spectra but we found that even this modified formula was insufficient to explain the relation between chemical shift and mineral structure. We therefore decided to investigate the effect of individual atoms in the structure on ^{29}Si

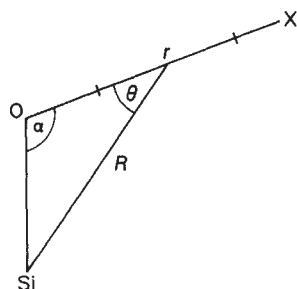


Fig. 1 Diagram showing the definitions of the angles θ and α , and the lengths r and R . X is any second-neighbour cation (including Si or Al).

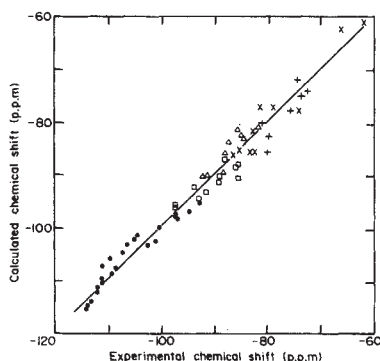


Fig. 2 Experimental chemical shift plotted against chemical shift calculated from equations (2) and (3) (x, orthosilicates; +, sorosilicates; Δ , inosilicates; $\square$, phyllosilicates; $\bullet$, tectosilicates).

chemical shift and have found a very significant correlation between the chemical shift and the orientation and valence of the cation-oxygen bonds immediately adjacent to the silica tetrahedron.

The measured NMR chemical shifts of 125 Si atoms in a wide range of silicates with known crystal structures were taken from various compilations^{1,2,4-8,10,14,15,17-20} and from our own unpublished spectra.

The Chem-X crystal structure modelling package (developed and distributed by Chemical Design Ltd, Oxford, UK) was used to examine X-ray and neutron diffraction crystal structures retrieved from the Inorganic Crystal Structure Database (ICSD) at CISTI in Ottawa²¹. Because the calculated chemical shifts described below depend indirectly on the Si-O distances it was necessary to exclude compounds with Al-Si disorder, as the T-O distances obtained from diffraction data in these cases are averages of Al-O and Si-O distances. Further compounds that showed discrepancies or uncertainties in either X-ray or NMR data were eliminated, leaving 76 data points in the final calculations: 29 from tectosilicates, 11 from orthosilicates, 6 from sorosilicates, 10 from inosilicates and 20 from phyllosilicates (Table 1).

Atomic coordinates from the ICSD were used to calculate the distance (R) between each silicon atom and the midpoint²² of the oxygen/second-neighbour-cation bonds, the length (r) of these bonds, the angle (θ) between R and r , and the Si-O-cation angle (α) (Fig. 1).

Initially the factor χ , given in equation (1), was calculated for the silica polymorphs from the magnetic anisotropy equation of McConnell²³:

$$\chi = \sum ((1 - 3 \cos^2 \theta_i) / 3R_i^3) \quad (1)$$

where the summation is over all the bonds between oxygen

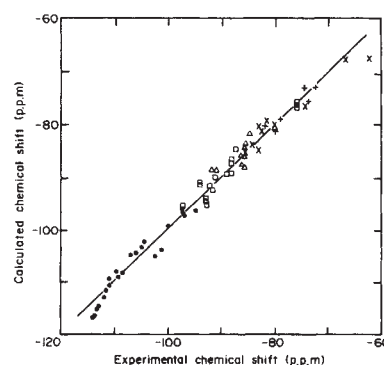


Fig. 3 Experimental chemical shift plotted against chemical shift calculated from equations (4) and (5) (x, orthosilicates; +, sorosilicates; Δ , inosilicates; $\square$, phyllosilicates; $\bullet$, tectosilicates).

atoms directly bonded to the silicon atom and the cations that are second neighbours to the silicon.

To use equation (1) for other silicate minerals it is necessary to take into account the variations in bond length and type of cation. This is done by multiplying each term in χ by the bond valence²⁴ s_i , given by $s_i = \exp [(r_0 - r_i) / 0.37]$, where values for r_0 are taken from Brown and Altermatt²⁴. This leads to a modified expression for χ :

$$\chi' = \sum s_i ((1 - 3 \cos^2 \theta_i) / 3R_i^3) \quad (2)$$

A regression of observed ²⁹Si chemical shift (δ) on χ' yields

$$\delta = 359.11\chi' - 44.37 \quad (3)$$

with a correlation coefficient of 0.985. The calculated chemical shifts shown in Fig. 2 were determined from equations (2) and (3).

Equation (3) was further refined to allow for the geometric distortions introduced by tight rings in the structure²⁵. Gibbs²⁶ 'hybridization index' for the bridging oxygen ($\cos \alpha / (\cos \alpha - 1)$) was found to give a useful correction. The result is

$$\chi'' = \sum s_i ((1 - 3 \cos^2 \theta_i) / 3R_i^3) (\cos \alpha_i / (\cos \alpha_i - 1)) \quad (4)$$

which gives the linear regression

$$\delta = 650.08\chi'' - 56.06 \quad (5)$$

with a correlation coefficient of 0.991. The calculated chemical shifts shown in Fig. 3 were obtained from equations (4) and (5). The standard deviation between the observed and calculated chemical shifts is 0.703 p.p.m.

In deriving equations (3) and (5), bonds to hydrogen were omitted partly because of uncertainty in the hydrogen position and partly because their omission led to better correlations.

The major contribution to the chemical shift is the orientation of the cation-oxygen bond²². As the θ varies from 0° to 90° the term $(1 - 3 \cos^2 \theta)$ varies from 1 to -2.

Equations (4) and (5) can be used to interpret ²⁹Si MAS NMR spectra particularly those with multiple peaks, which can be assigned unambiguously by calculating the NMR spectrum directly from the crystal structure.

The prediction of structure from NMR data is more difficult but it is possible to use equations (4) and (5) to assess the quality of structural data and to discriminate between different models, which is especially useful for those based on powder diffraction data. Because the technique only involves short-range calculations, it should be applicable to the study of amorphous solids, where the amount of information from diffraction experiments is extremely limited.

In minerals with Al-Si disorder, diffraction methods only determine the average of the Al-O and Si-O lengths. In zunyite, for example, the T1 site is estimated to contain 24% Al and the average length of the T(1)-O(2) length is 1.65 Å (ref. 27). We

Table 1 ^{29}Si chemical shifts

²⁹ Si NMR chemical shift (p.p.m.)						²⁹ Si NMR chemical shift (p.p.m.)					
Mineral	Measured	Calculated		References		Mineral	Measured	Calculated		References	
		from equations (2) and (3)	(4) and (5)	NMR	Structure			from equations (2) and (3)	(4) and (5)	NMR	Structure
Tectosilicates						Sorosilicates					
tridymite T5	-114.0	-115.1	-116.6	4	28	lawsonite	-81.0	-80.2	-80.5	10	39
tridymite T7	-113.5	-114.5	-116.2	4	28	hemimorphite	-80.0	-85.7	-80.5	10	40
tridymite T4	-113.0	-113.9	-115.0	4	28	rankinite T1	-74.5	-71.9	-73.2	8, 13	41
tridymite T2	-113.0	-113.9	-114.7	4	28	rankinite T2	-76.0	-76.4	-75.9	8, 13	41
tridymite T11	-112.0	-112.2	-112.7	4	28	akermanite	-73.7	-74.9	-75.8	8	42
tridymite T1	-111.5	-110.8	-111.5	4	28	gehlenite	-72.5	-74.3	-73.3	8	43
tridymite T10	-111.0	-110.5	-110.9	4	28						
tridymite T3	-111.0	-110.5	-110.8	4	28						
tridymite T9	-111.0	-110.1	-110.8	4	28						
petalite T1	-110.9	-107.3	-109.5	8	61	Inosilicates					
tridymite T12	-111.0	-110.0	-110.7	4	28	jadeite	-91.8	-90.1	-88.9	8	44
petalite T2	-109.5	-106.1	-107.9	8	61	spodumene	-91.4	-90.0	-90.1	8, 13, 17	45
tridymite T6	-109.3	-108.7	-109.1	4	28	tremolite T2	-91.2	-90.1	-88.9	8, 13, 17	46
tridymite T8	-109.3	-108.8	-109.1	4	28	elbaite	-88.1	-89.8	-89.3	17	47
cristobalite	-108.5	-107.8	-108.4	4	62	tremolite T1	-87.2	-84.2	-84.8	8, 13, 17	46
quartz	-107.1	-104.8	-104.9	4, 17	63	omphacite T1	-85.4	-84.9	-85.2	17	48
scapolite T1	-106.2	-103.2	-104.7	15	64	omphacite T2	-85.4	-81.5	-84.2	17	48
albite Amelia T2	-104.9	-102.1	-103.2	18	65	diopside T1	-84.7	-82.8	-82.1	8, 17	49
albite T2	-104.6	-101.6	-102.7	18	66	clinoenstatite T1	-84.2	-83.2	-84.0	8	50
beryl	-102.3	-103.4	-105.1	17	67	clinoenstatite T2	-81.8	-81.2	-80.6	8	50
beryl-Cs-Na	-101.0	-102.7	-103.6	17	68						
microcline T2	-100.2	-99.9	-99.4	18	69	Phyllosilicates					
albite T3	-97.1	-98.0	-97.1	18	66	talc T1	-97.2	-96.1	-95.6	13, 17	51
albite Amelia T3	-97.1	-97.4	-97.0	18	65	talc T2	-97.2	-95.9	-95.5	13, 17	51
microcline T3	-97.1	-97.9	-96.2	18	69	pyrophyllite T2	-94.0	-92.3	-91.2	13, 17	52
microcline T4	-94.7	-97.0	-96.6	18	69	pyrophyllite T1	-94.0	-92.1	-91.0	13, 17	52
albite T4	-92.8	-94.3	94.6	18	66	apophyllite	-92.0	-90.8	-91.7	13	53
albite Amelia T4	-92.8	-94.3	-94.3	18	65	kaolinite T1	-91.5	-93.3	-92.0	13, 17, 19	54
scapolite T2	-92.6	-95.3	-94.8	15	64	kaolinite T2	-91.5	-93.3	-92.1	13, 17, 19	54
						kaolinite T4	-91.5	-93.2	-91.9	13, 17, 19	54
Orthosilicates						kaolinite T3	-91.5	-93.1	-91.9	13, 17, 19	54
sillimanite	-86.4	-86.1	-86.1	8, 13, 17	29	tremolite T1	-91.2	-90.2	-88.9	8, 13, 17	46
topaz	-85.6	-85.5	-84.8	13	30	danburite	-89.0	-90.1	-89.6	17	55
kyanite T2	-83.2	-85.9	-85.1	17	31	lepidolite T2	-89.0	-91.5	-89.4	13, 17	56
datolite	-83.0	-81.6	-80.5	17	32	lepidolite T1	-89.0	-91.5	-89.4	13, 17	56
kyanite T1	-82.3	-85.8	-81.3	17	31	vermiculite T1	-88.0	-87.0	-87.4	20	57
zircon	-81.6	-77.1	-79.8	13	33	vermiculite T2	-88.0	-86.0	-86.7	20	57
andalusite	-79.8	-82.9	-81.2	8, 13, 17	34	phlogopite	-86.0	-88.7	-87.4	13, 20	58
spinel	-79.0	-77.3	-79.1	17	35	muscovite T1	-85.5	-90.6	-88.3	13, 17, 20	59
phenacite	-74.2	-78.0	-76.7	13	36	muscovite T2	-85.5	-88.0	-86.4	13, 17, 20	59
monticellite	-66.3	-62.2	-67.8	8	37	margarite T1	-76.0	-77.8	-77.0	13, 19	60
olivine	-62.0	-61.1	-67.8	13	38	margarite T2	-76.0	-76.9	-76.3	13, 19	60

have been able to use equations (4) and (5) to show that the probable Si (1)-O(2) distance is 1.56 Å, which is similar to that found in tridymite²⁸.

We are currently examining whether equations (4) and (5) can be applied to the interpretation of MAS NMR spectra of silicon-containing structures other than silicates, as well as to the interpretation of the spectra of other nuclei.

This work was funded by NSERC through a scholarship to B.L.S. and an operating grant to H.D.G. We also acknowledge helpful discussions with Professor J. S. Hartman of Brock University, St Catharines, and Professors M. J. McGlinchey and I. D. Brown of McMaster University.

Received 19 October 1987; accepted 11 January 1988.

- Lippmaa, E., Mägi, M., Samoson, A., Engelhardt, G. & Grimmer, A.-R. *J. Am. chem. Soc.* **102**, 4889-4893 (1980).
- Lippmaa, E., Mägi, M., Samoson, A., Tarmak, M. & Engelhardt, G. *J. Am. chem. Soc.* **103**, 4992-4996 (1981).
- Grimmer, A.-R., von Lampe, F., Mägi, M. & Lippmaa, E. *Mh. Chem.* **114**, 1053-1057 (1983); *Mh. Chem.* **115**, 561-564 (1984).
- Smith, J. V. & Blackwell, C. S. *Nature* **303**, 223-225 (1983).
- Engelhardt, G. & Radeglia, R. *Chem. Phys. Lett.* **108**, 271-274 (1984).
- Radeglia, R. & Engelhardt, G. *Chem. Phys. Lett.* **114**, 28-30 (1985).
- Ramdas, S. & Klinowski, J. *Nature* **308**, 521-525 (1984).
- Janes, N. & Oldfield, E. *J. Am. chem. Soc.* **107**, 6769-6775 (1985).
- Higgins, J. B. & Woessner, D. E. *Eos* **63**, 1139 (1982).
- Smith, K. A., Kirkpatrick, R. J., Oldfield, E. & Henderson, D. M. *Am. Miner.* **68**, 1206-1215 (1983).
- Grimmer, A.-R. & Radeglia, R. *Chem. Phys. Lett.* **106**, 262-265 (1984).
- Grimmer, A.-R. *Chem. Phys. Lett.* **119**, 416-420 (1985).
- Mägi, M., Lippmaa, E., Samoson, A., Engelhardt, G. & Grimmer, A.-R. *J. phys. Chem.* **88**, 1518-1522 (1984).

- Newsam, J. M. *J. phys. Chem.* **89**, 2002-2005 (1985).
- Sherriff, B. L., Grundy, D. G. & Hartman, J. S. *Can. Miner.* **25**, 717-730 (1988).
- Tossell, J. A. & Lazzarotti, P. *J. chem. Phys.* **84**, 369-374 (1986); *J. chem. Phys.* **112**, 205-212 (1987).
- Sherriff, B. L. thesis, Brock Univ. (1984).
- Sherriff, B. L. & Hartman, J. S. *Can. Miner.* **23**, 205-212 (1984).
- Kinsey, R. A., Kirkpatrick, R. J., Hawer, J., Smith, K. A. & Oldfield, E. *Am. Miner.* **70**, 537-548 (1985).
- Sanz, J. & Serratos, J. M. *Am. chem. Soc.* **106**, 4790-4793 (1984).
- Bergerhoff, G., Hundt, R., Sievers, R. & Brown, I. D. *J. chem. Inform. Comput. Sci.* **23**, 66-69 (1983).
- McGlinchey, M. J., Burns, R. C., Hofer, R., Top, S. & Jaouen, G. *Organometallics* **5**, 104-109 (1986).
- McConnell, H. M. *J. chem. Phys.* **27**, 226-229 (1957).
- Altermatt, D. & Brown, I. D. *Acta crystallogr.* **B41**, 240-244 (1985); Brown, I. D. & Altermatt, D. *Acta crystallogr.* **B41**, 244-247 (1985).
- Hoebbel, D. et al. *J. anorg. allg. Chem.* **424**, 115-127 (1976).
- Gibbs, G. V. *Am. Miner.* **67**, 421-450 (1982).
- Bauer, W. H. & Ohta, T. *Acta crystallogr.* **B38**, 390-401 (1982).
- Bauer, W. H. *Acta crystallogr.* **B33**, 2615-2619 (1977).
- Burnham, C. W. *Z. Kristallogr.* **118**, 127-148 (1963).
- Zobetz, E., Zemann, J., Heger, G. & Voellenkle, H. *Anz. Österr. Akad. Wiss. Math.-Naturwiss.* **Kl. 116**, 145-147 (1979).
- Burnham, C. W. *Z. Kristallogr.* **118**, 337-360 (1963).
- Foit, F. F. Jr, Phillips, M. W. & Gibbs, G. V. *Am. Miner.* **58**, 909-914 (1973).
- Robinson, K., Gibbs, G. V. & Ribbe, P. H. *Am. Miner.* **56**, 782-789 (1971).
- Burnham, C. W. & Buerger, M. J. *Z. Kristallogr.* **115**, 269-290 (1961).
- Speer, J. A. & Gibbs, G. V. *Am. Miner.* **61**, 238-247 (1976).
- Zachariasen, W. H. *Kristallografiya* **16**, 1161-1166 (1971).
- Onken, H. *Tschermaks Miner. Petrogr.* **10**, 34-44 (1965).
- Birle, J. D., Gibbs, G. V., Moore, P. B. & Smith, J. V. *Am. Miner.* **53**, 807-824 (1968).
- Baur, W. H. *Am. Miner.* **63**, 311-315 (1978).
- McDonald, W. S. & Cruickshank, D. W. Z. *Kristallogr.* **124**, 180-191 (1967).
- Saburi, S., Kusachi, I., Henmi, K. & Kawada, I. *Miner. J., Tokyo* **8**, 240-246 (1976).
- Kimeta, M. & Li, N. *Neues Jb. Miner. Abh.* **1-10** (1981).
- Kimata, M. & Li, N. *Neues Jb. Miner. Abh.* **143**, 254-267 (1982).
- Prewitt, C. T. & Burnham, C. W. *Am. Miner.* **51**, 956-975 (1966).
- Clark, J. R., Appleman, D. E. & Papike, J. J. *Miner. Soc. Am. spec. Pap.* **2**, 31-50 (1965).
- Hawthorne, F. C. & Grundy, H. D. *Can. Miner.* **14**, 334-345 (1976).
- Donnay, G. & Buerger, M. J. *Acta Crystallogr.* **3**, 379-388 (1950).

48. Matsumoto, T., Tokonami, M. & Morimoto, N. *Am. Miner.* **60**, 634-641 (1975).
49. Bruno, E., Carbonin, S. & Molin, G. *Tschermaks Miner. Petrogr.* **29**, 223-240 (1982).
50. Pannhorst, W., *Neues Jb. Miner. Abh.* **150**, 209-218 (1984).
51. Rayner, J. H. & Brown, G. *Clays Clay Minerals* **21**, 103-114 (1973).
52. Lee, J. H. & Guggenheim, S. *Am. Miner.* **66**, 350-357 (1981).
53. Prince, E. *Am. Miner.* **56**, 1243-1251 (1971).
54. Drits, V. A. & Kashaev, A. A. *Kristallografiya* **5**, 224-227 (1960).
55. Phillips, M. W., Gibbs, G. V. & Ribbe, P. H. *Am. Miner.* **59**, 79-84 (1974).
56. Guggenheim, S. *Am. Miner.* **66**, 1221-1232 (1981).
57. Shirozu, H. & Bailey, S. W. *Am. Miner.* **51**, 1124-1143 (1966).
58. Rayner, J. H. *Miner. Mag.* **39**, 850-856 (1974).
59. Gueven, N. & Burnham, C. W. *Carnegie Inst. Wash. Yb.* **65**, 290-293 (1966).
60. Iakeuchi, Y. *Clays Clay Minerals* **13**, 1-25 (1966).
61. Tagai, T., Ried, H., Joswig, W. & Korekawa, M. *Z. Kristallogr.* **160**, 159-170 (1982).
62. Dollase, W. A. *Z. Kristallogr.* **121**, 369-377 (1965).
63. Le Page, Y. & Donnay, G. *Acta crystallogr.* **32**, 2456-2459 (1976).
64. Levien, L. & Papike, J. J. *Am. Miner.* **61**, 864-877 (1976).
65. Harlow, G. E. & Brown, G. E. Jr *Am. Miner.* **65**, 986-995 (1980).
66. Ferguson, R. B. & Traill, R. J. *Acta crystallogr.* **25**, 1503-1517 (1969).
67. Morosin, B. *Acta crystallogr.* **28**, 1899-1903 (1972).
68. Hawthorne, F. C. & Cerny, P. *Can. Miner.* **15**, 414-421 (1977).
69. Brown, B. E. & Bailey, Y. W. *Acta crystallogr.* **17**, 1391-1400 (1964).

Thermodynamic stability of a smectic phase in a system of hard rods

D. Frenkel*, H. N. W. Lekkerkerker† & A. Stroobants†

* FOM Institute for Atomic and Molecular Physics, PO Box 41883, 1009 DB Amsterdam, The Netherlands

† Van 't Hoff Laboratory, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

One of the most remarkable phenomena exhibited by colloidal suspensions of monodisperse rod-like particles is the spontaneous formation of smectic liquid crystals¹⁻⁵. In these smectic phases, the particles order in periodic layers; on average, the axes of the rods are perpendicular to the layers. Smectics are distinct from crystals in that there is no long-range positional order within the layers. Because the spacing of the smectic layers is of the order of optical wavelengths, white light is separated into colours when scattered, giving rise to beautiful iridescence as in the colour photographs of ref. 2. As early as 1949, Onsager⁶ showed that nematic ordering may arise from hard-core repulsions between anisometric particles. However, it appears to have been generally accepted in the literature that smectic liquid crystalline ordering demands that attractive forces also operate⁷. There is nevertheless experimental evidence that smectic ordering does occur in colloidal systems where the particles interact predominantly through repulsive electrostatic interactions. This observation raises the fundamental question of whether smectic ordering can occur in a system of particles with purely repulsive interactions. Inspired by the seminal work of Alder and Wainwright⁸ on the freezing of a system of hard spheres, we have explored the possibility that smectic ordering occurs in a fluid of hard rod-like particles. Earlier computer simulations on hard parallel spherocylinders^{9,10} (cylinders with length L and diameter D , capped at each end with hemispheres of the same diameter) indicated that, in this somewhat artificial system, smectic order was indeed possible. Here we present numerical evidence that hard spherocylinders with both translational and orientational freedom can form a thermodynamically stable smectic phase.

Molecular dynamic calculations (MD) and constant pressure Monte Carlo simulations (MC) were done on a system of 576 hard spherocylinders with $L/D = 5$. Periodic boundary conditions were imposed to minimize the effect of finite system size. Initially the spherocylinders were arranged in a face-centred cubic crystal which was expanded along the [111] axis by a factor $1 + L/D$. The shape of the resulting simulation box was very nearly cubic. This close-packed lattice was then expanded to five times its original volume. At this low density the spherocylinders form a translationally and orientationally disordered

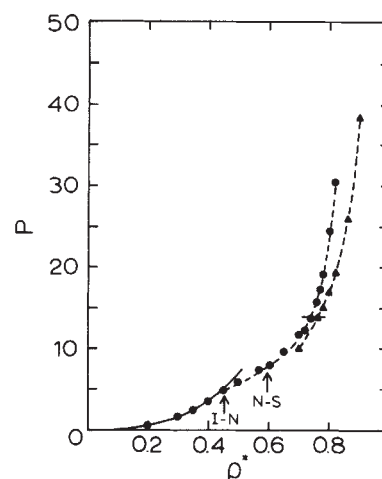


Fig. 1 Equation of state of hard spherocylinders with $L/D = 5$. The reduced density ρ^* equals ρ/ρ_{cp} , where ρ_{cp} is the density at regular close packing. The reduced pressure P^* is defined as Pv_0/kT , where v_0 is the volume per particle. The circles denote the molecular dynamics results for the pressure in the fluid phase, and the triangles correspond to the crystalline branch. The dashed curves are numerical fits to the data points. The drawn curve represents a five-term virial series, evaluated using the virial coefficients of this model system, which had been computed separately¹². The location of the isotropic-nematic and nematic-smectic phase transitions (see text) are indicated by arrows. The horizontal bar at $P^* = 13.8$ connects the densities of the coexisting smectic and crystalline phases.

cylinders form a translationally and orientationally disordered fluid. Visual inspection of snapshots of the arrangements of the particles and computation of the appropriate order parameters confirmed that the fluid at 20% of close packing was free of residual positional and orientational order. The fluid was slowly compressed to higher densities. Typically, the density was increased in steps of 5% of the close-packed density. At every new density the system was equilibrated for at least 10,000 trial moves per particle (cycles), followed by alternating MC and MD production runs. The typical length of the MD runs was $1-2 \times 10^6$ collisions and the MC runs consisted of 20,000 cycles. Close to phase transitions it was generally necessary to equilibrate for much longer. Figure 1 shows the equation-of-state data obtained in these simulations.

At a density corresponding to 45% of regular close packing, the system was observed to transform spontaneously into an orientationally ordered fluid. Analysis of the long-range orientational and positional correlations in this phase indicated that it was in fact a nematic liquid crystal.

Upon further compression an increase was noted in the amplitude of a particular Fourier component of the density fluctuations, namely the one with a wave-vector of order $2\pi/(L+D)$ pointing along the nematic director. As the density increased, the amplitude of this Fourier component grew, as did its decay time. At 60% of close packing, the system developed a static one-dimensional density modulation along the director, but no translational ordering was observed in the directions perpendicular to the director. Such one-dimensional ordering is the hallmark of a smectic-A liquid-crystalline phase. A typical example of the instantaneous arrangement of the particles in this phase is shown in Fig. 2. Although the fact that the latter phase formed spontaneously on compression indicates that it is stable with respect to both the isotropic and the nematic phases, its thermodynamic stability with respect to the crystalline state remained to be established. To this end we computed the

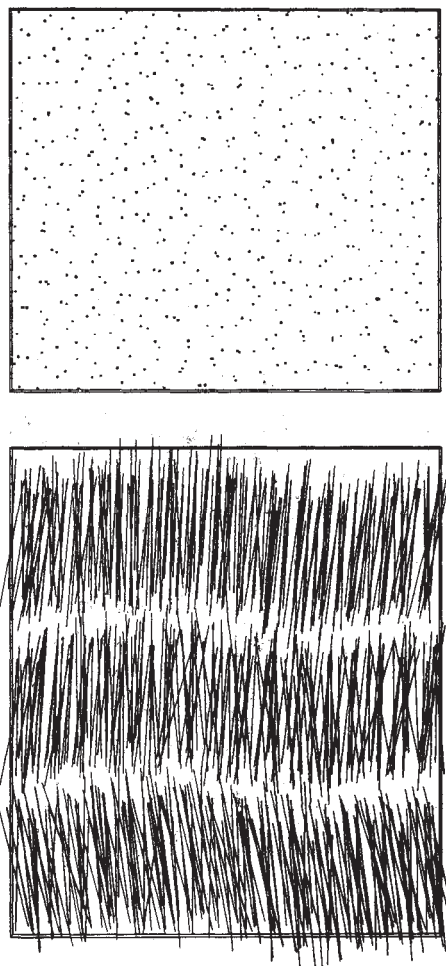


Fig. 2 Typical example of a configuration of 576 hard spherocylinders in the smectic-A phase ($\rho^* = 0.65$). The lower figure shows a side view of the orientationally ordered fluid. For clarity, the spherocylinders are represented by line segments of length L . The upper figure shows a projection of the centres of mass of the particles (dots) on a plane perpendicular to the direction of orientational alignment. Note that there is no translational order in this plane.

chemical potential of the solid phase at a reduced density of 0.86 using the techniques described in ref. 11. The equation-of-state of the crystalline solid was obtained by (constant-stress) MC and MD simulation on systems of 144 spherocylinders with density between 70% and 90% of close packing. The chemical potential of the fluid phase was obtained by thermodynamic integration from the dilute gas. This integration of the fluid equation-of-state was achieved by fitting the numerical equation-of-state data to a polynomial in $\phi/(1-\phi)$, where ϕ is the volume fraction. The polynomial was integrated analytically. The fluid-solid coexistence point was then found by locating the densities at which the pressure and chemical potential of the two phases were equal. Using this procedure we found that a smectic phase with a density of 73% of close packing coexists with a crystalline solid with a density of 76% of close packing.

The nematic to smectic transition is accompanied by pre-translational fluctuations usually associated with a continuous phase transition, but the system size used did not allow us to distinguish unambiguously between a continuous phase transition and a weakly first-order one. The phase transition from the smectic phase to the crystalline solid is first order. The jump in the density at this transition is $\sim 4\%$, and should be compared with a value of $\sim 10\%$ for the corresponding quantity at the

fluid-solid transition in a hard sphere system. Finally, it is amusing to note that in the system studied here the width of the range of stability of the 'unexpected' smectic phase is comparable with that of the nematic phase.

This work is part of the research programme of Foundation for Fundamental Research of Matter (FOM) with financial support from Netherlands Organisation for Pure Research (ZWO).

Received 20 November 1987; accepted 27 January 1988.

1. Oster, G. *J. gen. Physiol.* **33**, 445-473 (1950).
2. Kreibitz, U. & Wetter, C. *Z. Naturforsch.* **35C**, 750-762 (1980).
3. Fraden, S., Hurd, A. J., Meyer, R. B., Cahoon, M. & Caspar, D. L. *J. Phys. (Paris), Colloq.* **46** C3, 85-113 (1985).
4. Maeda, Y. & Hachisu, S. *Colloids and Interfaces* **6**, 1-16 (1983).
5. Maeda, Y. & Hachisu, S. *Colloids and Interfaces* **7**, 357-360 (1984).
6. Onsager, L. *Ann. N.Y. Acad. Sci.* **51**, 627-659 (1949).
7. Kloczkowski, A. & Stecki, J. *Molec. Phys.* **55**, 689-700 (1985).
8. Alder, B. J. & Wainwright, T. E. *J. Chem. Phys.* **27**, 1208-1209 (1957).
9. Stroobants, A., Lekkerkerker, H. N. W. & Frenkel, D. *Phys. Rev. Lett.* **57**, 1452-1455 (1986).
10. Stroobants, A., Lekkerkerker, H. N. W. & Frenkel, D. *Phys. Rev. A* **36**, 2929-2945 (1987).
11. Frenkel, D. & Mulder, B. M. *Molec. Phys.* **55**, 1171-1192 (1985).
12. Frenkel, D. *J. phys. Chem.* (in the press).

Majorite fractionation recorded in the geochemistry of peridotites from South Africa

Claude Herzberg*, Mark Feigenson*, Cherylyn Skuba† & Eiji Ohtani‡

* Department of Geological Sciences, Rutgers University, New Brunswick, New Jersey 08903, USA

† Department of Geology, University of Texas at Arlington, Arlington, Texas 76019, USA

‡ Department of Earth Sciences, Ehime University, Matsuyama 790, Japan

The liquidus phase for ultrabasic rock compositions changes from olivine at low pressures to a pyroxene polymorph at high pressures^{1,2}. In the range 13-25 GPa, the liquidus phase for a chondritic bulk Earth composition is majorite^{3,4}, a pyroxene with the garnet crystal structure. For upper-mantle lherzolite compositions, majorite is also the liquidus phase from ~ 16 -25 GPa⁵⁻⁷, and transforms to perovskite at similar or higher pressures⁷. As majorite has a density higher than peridotite magmas^{8,9}, it could have fractionated during an early differentiation event in the Earth³. Here we argue that this process is recorded in the major-element geochemistry of peridotites from South Africa, and that it has resulted in a lower mantle enriched in silica.

Figure 1a shows in projection the major-element geochemistry of a world database for peridotites. The compilation of Maaloe and Aoki¹⁰ has been used ($N = 504$), and upgraded in this study to include more recent analyses ($N = 1,040$). There is a clear bifurcation in this plot, with most peridotites from South Africa occupying the right-hand trend and most peridotites from the rest of the world occupying the left-hand trend. For the purpose of clarification, the two populations of data are plotted separately in Fig. 1b (world database excluding samples from South Africa) and Fig. 1c (samples from South Africa). The South African samples from the continental lithosphere consist largely of the orthopyroxene-rich granular garnet lherzolite and garnet harzburgite xenoliths. The fertile sheared garnet lherzolites from South Africa, which are not as abundant and are thought to be pieces of asthenosphere¹¹, are plotted in the left-hand trend defined mainly by spinel peridotites from the rest of the world. Harzburgite residua from ophiolite complexes occupy the point of the V in Fig. 1a.

Superimposed on the geochemistry of mantle peridotite are the locations of the experimentally constrained primary phase

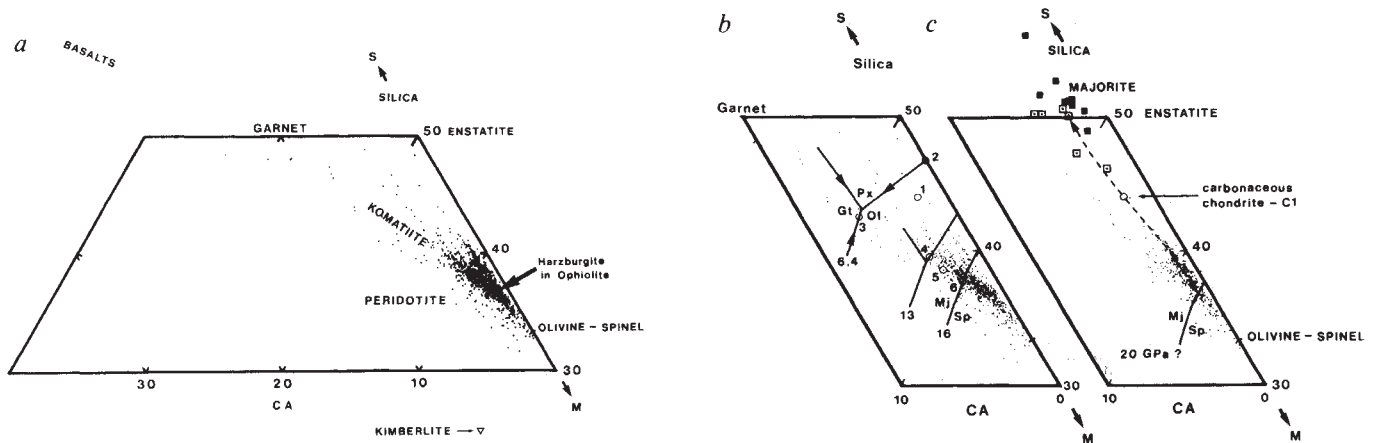


Fig. 1 *a*, A world database for upper-mantle peridotite (dots) projected from diopside into the plane CA-M-S after O'Hara²⁹ (mole %), compared with basalt (average mid-ocean ridge basalt glass³⁰), kimberlite³¹ and komatiite². *b*, Mantle peridotites from the world, excluding samples from South Africa, and experimentally constrained primary-phase fields on the following compositions (open circles): no. 1 (carbonaceous chondrite with $Mg/(Mg + Fe) = 0.9$)³; no. 2 (ref. 17); no. 3 (komatiite)¹³; no. 4 (lherzolite)¹⁴; no. 5 (pyrolite)¹²; no. 6 (lherzolite; KLB1 and 1611)^{5-7,15,16}. Pressures of the equilibria are in GPa. The thermal minimum at 16 GPa must be located along the majorite-spinel $(Mg, Fe)_2SiO_4$ multiple saturation surface at CA contents slightly less than bulk composition no. 6, and has an MgO content of about 38%^{5,7}. *c*, Mantle peridotites from South Africa, and a majorite fractionation path from a chondritic bulk Earth (broken curve). Open squares with dots are the majorite analyses of Ohtani and others^{3,4} on the C1 carbonaceous chondrite composition shown ($Mg/(Mg + Fe) = 0.9$; bulk composition no. 1 in *b*). Majorites nearest the liquidus are plotted to the left, and those nearest the solidus are plotted to the right. The reconstructed orthopyroxene analyses of Cox *et al.*²³ (solid squares) are interpreted to be largely recrystallized majorite. Large samples (>500 g)^{21,23} in the trend are almost parallel to the S-M join.

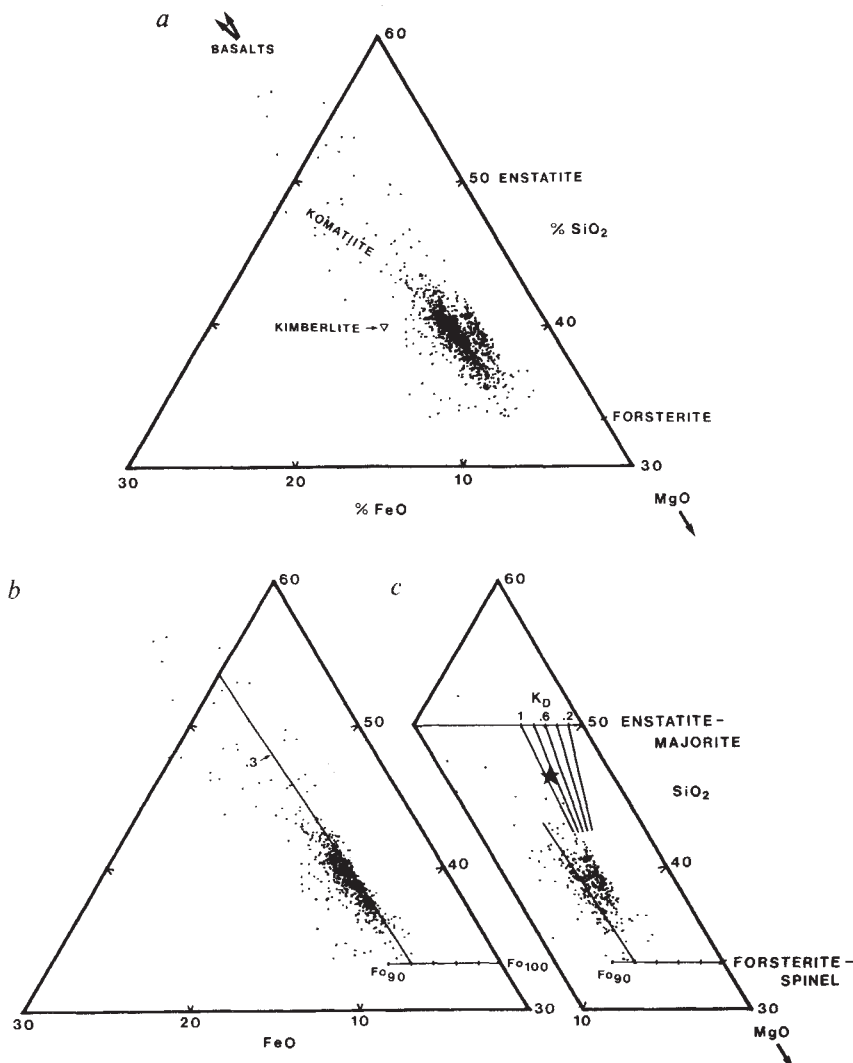
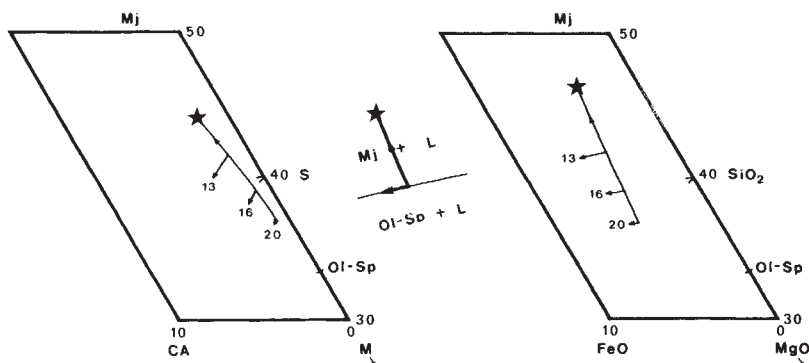


Fig. 2 *a*, FeO-MgO-SiO₂ relations (mole %) for mantle peridotite. *b*, Mantle peridotites from the world excluding South Africa. Equilibrium olivine (Fo_{92}) and liquid pairs are plotted on or to the left of the line indicated as 0.3, the Fe-Mg partition coefficient

$K_D = (MgO/FeO)_{liquid} \cdot (FeO/MgO)_{olivine}$ for basalts. Harzburgites and serpentinites from ophiolites are confined to this plot. *c*, Mantle peridotites from South Africa. The far right-hand trend is defined by the refractory granular types; a chondritic mantle (solid star) having $MgO/(MgO + FeO) = 0.92-0.93$ is deduced from chondritic Mg/Si. Harzburgites and serpentinites from ophiolites are excluded from the right-hand trend. Some granular types also scatter to the left in the main world trend with the sheared types. Lines radiating toward majorite orient coexisting majorite-liquid peridotite pairs with the partition coefficients shown; a value of 0.6 to 1.0 is deduced, consistent with garnet-liquid equilibria at low pressure³². The line from Fo_{92} bounds equilibrium olivine-melt pairs with a partition coefficient of 0.3 (*b*); the right-hand trend does not parallel this line, demonstrating that these rocks cannot be residues.

Fig. 3 A crystallization-differentiation model for mantle peridotite from a chondritic Earth (solid star). Equilibrium fusion and crystallization paths are indicated at the approximate pressures of multiple saturation in majorite (Mj) and olivine (Ol) or modified spinel (Sp) (L + Mj + Ol-Sp). In CMAS (left), liquids having the composition of spinel peridotite are located at or near thermal minima (Fig. 1b). In FeO-MgO-SiO₂ (right), these liquids reside at or, more probably, to the Mg-rich side of thermal minima that extend further toward FeO. Fractionation of majorite and modified spinel could have produced a silica-rich lower mantle that was also somewhat higher in Mg/(Mg + Fe) than the upper mantle.



fields for minerals that crystallize from magmas at very high pressures^{3-7,12-17} (Fig. 1b). 'Fertile' peridotites with low MgO (that is, 36-38 wt%) in the main world trend have the properties of liquids formed at or near pseudoquaternary eutectic-like equilibria and thermal minima, supporting the hypothesis that the upper mantle formed from the solidification of these liquids². The high-MgO peridotites (>38%) may be either minimum melt compositions formed at pressures >16 GPa, or residua remaining after basalt extraction. Indeed, even low-MgO peridotites have a rare-earth-element geochemistry showing melt removal¹⁸, indicating that Fig. 1b cannot be used at present to discriminate solidified minimum melts from residua.

The refractory character of the granular peridotites from South Africa has long been noted^{10,11,19}, and is shown by the low contents of CaO and Al₂O₃ (CA in Fig. 1), the basalt-producing components. From this observation the conclusion was drawn that these peridotites are residues left after the extraction of basalt^{11,19} or komatiite. But Maaloe and Aoki¹⁰ and others have pointed out that it is impossible to relate residual and fertile peridotites by addition or subtraction of basalt or kimberlite (komatiite is also included), and this is shown in Fig. 1a. The residuum hypothesis is therefore fundamentally flawed.

The South African trend is collinear with carbonaceous chondrite and the majorite analyses of Ohtani and coworkers^{3,4}. We interpret the South African trend to be a record and majorite fractionation that occurred when peridotite liquids were multiply saturated in majorite and modified spinel. This probably occurred over a range of pressures because the trend suggests a shift in the 16-GPa thermal minimum (38 wt% MgO) to peridotite liquids with ~44% MgO, possibly becoming stable at ~20 GPa. For a bulk Earth with the composition of C1 carbonaceous chondrite, the amount of majorite fractionation required to explain the South African trend is 50-70% by mass.

Variations in Ca/Al cannot be seen in Fig. 1. However, it has been pointed out that Ca/Al for most spinel peridotites is usually greater than chondritic²⁰, and we have observed this in our database for the main world trend as well. The experimental data of Ohtani *et al.*³ show that this is consistent with Ca/Al of peridotite liquids in equilibrium with majorite and modified spinel at and near the solidus. But many of the granular peridotites from South Africa have Ca/Al less than chondritic. Addition of either majorite or modified spinel to peridotite liquids is strongly indicated because both have Ca/Al equal to or less than chondritic³. We therefore interpret the granular South African peridotites to be solidified crystal mushes of cumulus majorite and/or modified spinel and intercumulus liquid peridotite, formed probably near the solidus.

FeO-MgO-SiO₂ relations in the peridotite world database also show two distinct trends (Fig. 2a). It has been noted previously^{11,21} that most granular lherzolites from South Africa are higher in MgO/(MgO + FeO) than peridotites from the rest of the world, and this is demonstrated in Fig. 2b,c. The residuum hypothesis fails again because the 'refractory' South African trend cannot be produced from the 'fertile' trend by the subtraction of basalt, komatiite or kimberlite.

tion of basalt, komatiite or kimberlite.

The main world trend (Fig. 2b) shows an increase in MgO/(MgO + FeO) with increasing olivine content of the rocks. The data are consistent with equilibrium partitioning of Fe and Mg between olivine and basaltic magmas, and record a history of partial melting and basalt extraction.

The residuum and solidified-melt hypotheses for the South African peridotites predict very different Fe-Mg relations. If these are residual peridotites, they should also display equilibrium Fe-Mg relations between olivine and liquid. Conversely, majorite fractionation should impose FeO enrichment on solidified peridotite liquids, with MgO/(MgO + FeO) actually decreasing with increasing olivine content. Indeed, the data are consistent with iron enrichment (fig. 2c), even though the samples probably contained modified spinel as a cumulus phase. In addition, the residuum hypothesis for the South African peridotites fails again because the trend does not follow an equilibrium olivine-melt control.

The Fe-Mg partition coefficient

$$(K_D = (\text{MgO}/\text{FeO})_{\text{liquid}} \cdot (\text{FeO}/\text{MgO})_{\text{majorite}})$$

for coexisting majorite and liquid can be deduced from Fig. 2c to be between about 0.6 and 1.0. Additions of modified spinel would account for samples having very high MgO/(MgO + FeO), indicating that a value of 1.0 is somewhat too high. These partition coefficients are consistent with experimental data on Fe-Mg between garnet and picritic melts at 2.5 GPa ($K_D = 0.91$)³², but are inconsistent with ultrahigh pressure data on majorite-liquid pairs^{3,4} that show K_D to be around 0.3. These low values are unambiguously in error because FeO in the liquid has been reduced to Fe metal in these experiments via reaction with carbon containers; metallic iron is usually reported in these experiments^{3,4,6}, and Fe-Mg mass balance amongst the silicate phases in the experiments on chondrite is not possible. Finally, we calculate K_D to be between 0.48 and 0.8 based on Fe-Mg partitioning between subsolidus olivine and majorite at 13-15 GPa¹² and olivine-liquid values that range from 0.3 to 0.5³³, consistent with those deduced from Fig. 2c.

Figure 3 shows a crystallization-differentiation interpretation of the major-element geochemistry of mantle peridotite from a chondritic Earth. At pressures where liquidus majorite is stable, the crystallization sequence is: L, L + Mj, L + Mj + Sp, Mj + Sp. It can be inferred that most peridotites from the world are more fertile in the basalt-producing components CaO, Al₂O₃ and FeO than the granular South African types because they are either later-stage differentiates or are earlier partial fusion products of an early differentiating mantle. Peridotite liquids could not have developed high CaO, Al₂O₃ and FeO by majorite fractionation alone; co-precipitation of olivine or modified spinel would have been required (L + Mj + Sp). The most 'fertile' peridotites (for instance 36-38% MgO) could have formed by crystallization at relatively low pressures (for instance 13-16 GPa), resulting in ~50% majorite fractionation. Peridotites from South Africa with the highest silica contents could have crystallized in this

low pressure range as well; however, those with high MgO would have required up to 70% majorite fractionation, and this could have occurred at ~20 GPa.

Solidification of peridotite crystal mushes in the South African trend at ~16–20 GPa would have produced a biminerallitic rock consisting of majorite + modified spinel. Convective transport of this rock up to its present-day location in the continental lithosphere would have displaced it outside the stability fields of majorite and modified spinel, transforming majorite into orthopyroxene (moderately aluminous and calcic) and modified spinel (with some alimina³) into olivine. At temperatures and pressures within the South African lithosphere, the orthopyroxene compositions are not stable²², and exsolution of garnet and clinopyroxene would have occurred. Cox *et al.*²³ provided textural evidence supporting an exsolution origin for most of the clinopyroxenes and garnets. Their reconstructed orthopyroxenes, determined by adding garnet and clinopyroxene back into orthopyroxene, are very similar in composition to the majorites synthesized in the experiments on a chondritic bulk Earth^{3,4}. We therefore conclude that the precursor phase to the pyroxenes and garnets currently found in the granular South African peridotites was actually majorite, although modified spinel could have contributed some alumina to pyroxene as well.

The majorite fractionation hypothesis also predicts LREE-enrichments and HREE-depletions in peridotite liquids formed from a chondrite Earth in a single stage. Indeed, these characteristics are typical of the granular peridotite xenoliths from South Africa²⁸. Of course, these LREE-enrichments and HREE-depletions are usually attributed to metasomatism of residual peridotite. We do not contest metasomatic contributions to either the rare-earth element^{34–36} or even the major element^{36,37} geochemistry of mantle peridotite. However, we have been able to show that a simple and quantitative crystallization-differentiation model successfully explains the REE contents of a spectrum of mantle peridotite³⁸, indicating that explanations based strictly on metasomatism are likely to be too restrictive. Furthermore, metasomatism of the major elements from kimberlite can also explain some of the data that scatter toward kimberlite in Figs 1 and 2; however, its overall contribution to the major element geochemistry of the mantle is likely to be small.

It is reasonable to expect that majorite fractionation occurred by gravitational removal into what is now the Earth's lower mantle. A lower mantle containing a significant cumulate/residuum component of majorite or, more correctly, majorite transformed to perovskite, would be considerably enriched in silica compared with the upper mantle. This is a plausible answer to the geochemical problem of maintaining both a chondritic Earth and a peridotite upper mantle with higher Mg/Si, and is an alternative to the silica volatilization hypothesis of Ringwood²⁴. Another problem is how these geochemical differences are geophysically structured. In the whole-mantle convection model, perovskite may have to become concentrated somewhere and decoupled from the mantle flow because its geochemical signature is not obvious in any mantle rocks studied so far. In the two-layer convection model, silica enrichment stemming from majorite fractionation might occur at the 670-km discontinuity, a model similar to those proposed by others^{25–27}.

We thank S. Bergman, F. R. Boyd, F. Frey, S. Haggerty, S. Maaloe, P. Nixon, C. Scarfe, D. Walker and especially K. G. Cox for critical comments.

Received 9 November 1987; accepted 15 March 1988.

1. Herzberg, C. T. *Phys. Earth Planet. Inter.* **32**, 193–202 (1983).
2. Herzberg, C. T. & O'Hara, M. J. *Geophys. Res. Lett.* **12**, 541–544 (1985).
3. Ohtani, E. *et al.* *Nature* **323**, 352–353 (1986).
4. Ohtani, E. & Sawamoto, H. J. *Geophys. Res. Lett.* **14**, 733–736 (1987).
5. Takahashi, E. J. *Geophys. Res. Lett.* **9**, 9367–9382 (1986).
6. Kato, T. & Kumazawa, M. *Geophys. Res. Lett.* **13**, 181–184 (1986).
7. Ito, E. & Takahashi, E. *Nature* **328**, 514–517 (1987).
8. Herzberg, C. T. in *Silicate Melts* (ed. Scarfe, C. M.) 279–304 (Mineral. Ass. Can. Short Course, 1986).

9. Ohtani, E. *Earth planet. Sci. Lett.* **67**, 261–272 (1984).
10. Maaloe, S. & Aoki, K.-i. *Contr. Miner. Petr.* **63**, 161–173 (1977).
11. Boyd, F. R. in *Mantle Xenoliths* (ed. Nixon, P. H.) 403–412 (Wiley, New York, 1987).
12. Nagata, Y., Ohtani, E. & Sawamoto, H. *28th High Pressure Conference of Japan, Kobe*, 114–115 (1987).
13. Herzberg, C. T. *et al.* *Eos* **67**, 408 (1986).
14. Herzberg, C. T. *Eos* **68**, 451 (1987).
15. Takahashi, E. & Scarfe, C. M. *Nature* **315**, 566–568 (1985).
16. Scarfe, C. M. & Takahashi, E. *Nature* **322**, 354–356 (1986).
17. Kato, T. & Kumazawa, M. J. *Phys. Earth* **33**, 513–524 (1985).
18. Frey, F. A. & Prinz, M. *Earth planet. Sci. Lett.* **38**, 129–176 (1978).
19. Nixon, P. H. & Boyd, F. R. in *Lesotho Kimberlites* (ed. Nixon, P. H.) 48–56 (Lesotho National Development Corporation, Lesotho, 1973).
20. Palme, H. & Nickel, K. G. *Geochim. cosmochim. Acta* **49**, 2123–2132 (1985).
21. Boyd, F. R. & Mertzman, S. A. in *Magmatic Processes: Physicochemical Principles* (ed. Mysen, B. O.) 13–24 (Geochemical Society, Spec. Publ. 1, Lancaster, 1987).
22. Finnerty, A. A. & Boyd, F. R. in *Mantle Xenoliths* (ed. Nixon, P. H.) 389–402 (Wiley, New York, 1987).
23. Cox, K. G. *et al.* in *Mantle Xenoliths* (ed. Nixon, P. H.) 537–550 (Wiley, New York, 1987).
24. Ringwood, A. E. *Origin of the Earth and Moon* (Springer-Verlag, 1979).
25. Liu, L.-g. *Geophys. Res. Lett.* **9**, 124–126 (1982).
26. Anderson, D. L. *Spec. Pap. Miner. Soc. Am.* **3**, 85–93 (1970).
27. Kumazawa, M. & Fukao, Y. *Int. Geodynamics Conf. Tokyo*, 276–277 (1978).
28. Shimizu, N. *Earth planet. Sci. Lett.* **25**, 26–32 (1975).
29. O'Hara, M. J. *Earth Sci. Rev.* **4**, 69–113 (1968).
30. Melson, W. G. *et al.* in *Geophysics of the Pacific Ocean Basin and its Margin*, 351–367 (American Geophysical Union, Washington, DC, 1976).
31. Wedepohl, K. H. & Maramatsu, Y. in *Kimberlites, Diatremes and Diamonds* (eds Boyd, F. R. & Meyer, H. O.) 300–312 (American Geophysical Union, Washington, DC, 1979).
32. Elthon, D. & Scarfe, C. M. *Am. Miner.* **69**, 1–15 (1984).
33. Herzberg, C. T. in *Magmatic Processes: Physicochemical Principles* (ed. Mysen, B.) 47–58 (Geochem. Soc. Spec. Publ. 1, 1987).
34. Harte, B. *et al.* in *Mantle Metasomatism* (eds Menzies, M. A. & Hawkesworth, C. J.) 145–220 (Academic, London, 1987).
35. Haggerty, S. E. *et al.* *Am. Miner.* **68**, 494–505 (1984).
36. Feigenson, M. D. *Geophys. Res. Lett.* **13**, 965–968 (1986).
37. Smith, D. & Boyd, F. R. in *Mantle Xenoliths* (ed. Nixon, P. H.) 551–561 (Wiley, New York, 1987).
38. Feigenson, M. D. *et al.* *Trans. Am. geophys. Un.* in the press.

The impact of a single black snowfall on streamwater chemistry in the Scottish Highlands

M. Tranter*, P. W. Abraham†, I. L. Blackwood, P. Brimblecombe & T. D. Davies

School of Environmental Sciences, University of East Anglia, Norwich, Norfolk NR4 7TJ, UK

* Department of Oceanography, The University, Southampton SO9 5NH, UK

During snowmelt, ions fractionate into the first meltwaters^{1–3}, giving rise to concentrated, often acidic, solutions^{4,5}. This may cause transient acidification of streamwaters (the so-called 'acid flush') in poorly buffered upland catchments⁶, which can kill fish⁷ or, more commonly, cause recruitment failure⁸. The chemistry of snow and meltwater can affect the magnitude and duration of the 'acid flush', particularly when stream discharge is not dominated by pre-event water. Such conditions are found in upland Britain, in the Scottish Highlands^{9,10}. The most acidic snowfalls in the Highlands are coloured or black¹¹, and occur under specific meteorological conditions¹². Here we present the streamwater chemistry during two acid-flush events with minimum recorded pH of 4.25 and 4.10. An acidic black snow (pH 3.4) fell in the 8-day period between the events. The meltwater composition during the first event was influenced by the composition of the existing snow-cover, whereas the second event shows the effect of the fall of polluted snow. These observations demonstrate that a single snowfall can strongly influence the composition of meltwaters. Hence, the large-scale distribution of ions in snowpacks must be considered in attempting to predict the magnitude of acidification during the thaw¹³ and the possible impact of acid rain on aquatic ecosystems. This work highlights how chance associations of episodic events (coloured snowfall and snowmelt) may amplify acidification in streamwater.

† Present address: Department of Geography, University College of Wales, Aberystwyth SW23 3DB, UK.

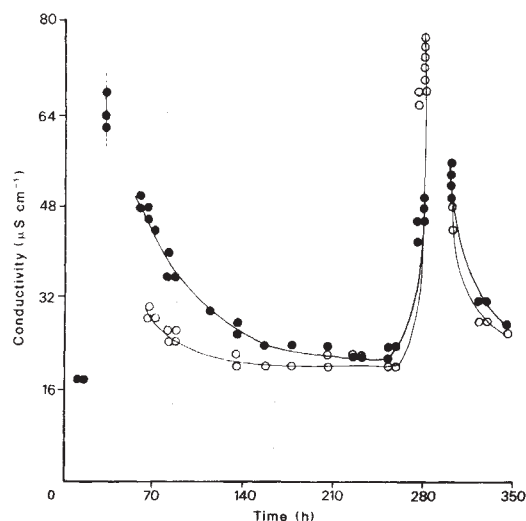


Fig. 1 The conductivity of streamwaters at site 1 (filled circles) and site 2 (open circles) from 6 to 18 March. Time zero is 00.00 h, 6 March. Two events of high conductivity ('acid flushes') are recorded.

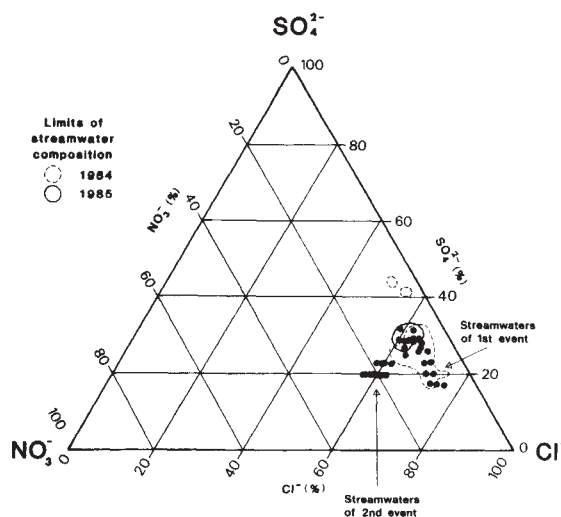


Fig. 2 The proportional anion composition of streamwaters at site 1. The streamwaters of the first event occupy similar positions to those sampled during the 1984 ($n=78$) and 1985 ($n=38$) seasons. Overlying points have not been indicated. Points are derived from data with units of equiv. l^{-1} . The sulphate-rich samples of 1984 were collected during late autumn.

The study area, Ciste Mhearad (Ordnance Survey National Grid NJ013046), is a remote catchment in the Scottish Highlands⁴. It has an area of $\sim 0.4 \text{ km}^2$ and an elevation of 1,010–1,160 m. The bedrock is granite, overlain by thin soils, classified as Alpine (humus ranker) and Alpine podzols¹⁴. The vegetation is Alpine tundra¹⁵. A single stream drains the catchment¹⁶.

The first thaws of 1986 occurred during 4–18 March, when two acid-flush events were recorded in the streamwaters. Samples of streamwater and meltwater were collected as frequently as weather conditions and resources allowed. Streamwater was sampled on all days at site 1, near the mouth of the catchment where the adjacent snowcover was $\sim 5 \text{ m}$ thick, and during 6–18 March at site 2, located in the bowl of the catchment where the thickness of snowcover was $\sim 3 \text{ m}$. Three snowpits at the same location were sampled on 12 January, 8 March and 9 March to determine snowpack composition. Black snow was collected at site 2 after the overnight fall on 13–14 March. A layer of snow, on average 2 cm deep (density $\sim 200 \text{ kg m}^{-3}$), had collected in the bowl of the catchment and had been subject to some wind redistribution, such that local thicknesses approached 1 m (I.L.B., in preparation). Meltwaters within the

snowpack were collected from three ledges in the side of a snowpit^{5,17,18}, $\sim 20 \text{ m}$ from site 2, on 15–17 March. Procedures for sample collection, treatment and storage are described elsewhere^{4,20,21}.

Figure 1 shows the stream conductivity records at both sites. Two events of high conductivity can be seen. Accompanying discharge data are not available. Previous work demonstrates that the rise in conductivity is accompanied by a rise in discharge¹⁶, and that snowmelt, rather than groundwater, is a dominant component of the bulk streamwater in this catchment during these events^{9,10}. Hence two acid-flush events are shown. Table 1 gives streamwater compositions at the maximum measured conductivity during each event and at background values. The difference in the concentrations of ions at each site may be the result of the differing contributions of groundwater, meltwater and soilwater to the bulk streamwater, and it is also likely that the composition of snowmelt and soilwater differs at each site (I.L.B., in preparation and ref. 22).

Table 1 Range of concentration of ions in streamwaters before and during both events

Date	Comment	Cond	pH	Cl	SO ₄	NO ₃	Na	K	Ca	Mg	Al	Pb	Mn	Si
Site 1														
4 March	Background	17	*	*	*	*	70	4	11	17	110	<1	3.0	*
5 March	Maximum measured during first event	67	4.2	440	95	41	340	12	33	86	410	2	8.0	2.1
14 March	Background before second event	22	4.7	85	42	12	87	4	13	22	130	1	4.0	1.7
16 March	Maximum measured during second event	55	4.3	260	110	95	200	10	25	52	450	3	12	1.8
Site 2														
14 March	Background before second event	20	4.6	68	38	13	76	4	6	19	90	2	3.0	1.8
15 March	Maximum measured during second event	78	4.0	170	170	100	150	16	16	43	880	9	13	2.0

Major cations were determined by atomic absorption spectroscopy, major anions by ion chromatography and trace metals by graphite furnace (except for Si, by the molybdenum blue method); pH was determined electrochemically.

Units: conductivity in $\mu\text{S cm}^{-1}$; Cl, SO₄, NO₃, Na, K, Ca, Mg in $\mu\text{equiv. l}^{-1}$; Al, Pb, Mn in $\mu\text{g l}^{-1}$; Si in mg l^{-1} .

*Unavailable.

Table 2 Concentrations of anions (in $\mu\text{equiv. l}^{-1}$) and pH of snowpack

Date	Min	pH Mean	Max.	Min.	Cl Mean	Max.	Min.	SO ₄ Mean	Max.	Min.	NO ₃ Mean	Max.	n
12 January	*	*	*	37	140	280	6	32	109	3	19	40	13
8 March	3.5	3.7	4.1	60	210	380	41	73	95	9	26	45	7
9 March	3.6	3.9	4.5	46	140	370	10	40	82	2	14	56	13
13-14 March		3.4			39			260			200		1
Black snow													

* Unavailable.

In Fig. 2, the anionic compositions of streamwaters at site 1 are compared with those collected during 1984 and 1985. The 1984-85 streamwaters include five melt events and the year's background condition²¹. During the first melt event, the anionic compositions resemble the 1984-85 samples. In contrast, the second event produced the most nitrate-rich samples collected in three years of study. We argue that the major anion concentrations and the pH during the second event were strongly influenced by the meltwater derived from one snow layer, the freshly fallen polluted snow. Therefore links between snow, meltwater and streamwater chemistry are examined.

Figure 3a and Table 2 give the anionic composition of snows near site 2 before and between the two events. The snows are predominantly chloride-rich, because marine aerosol is a major contributor to the solute load of the snowpack⁴. Most snows are more enriched in chloride than streamwaters before the second event. Although sulphate and nitrate tend to be preferentially lost from snowcover^{2-5,22}, the high proportion of chloride in the existing snowcover means that the first snowmelt is likely to be dominated by chloride⁵. The black snowfall of 13-14 March gave rise to a surface snow with higher concentrations and proportions of sulphate and nitrate than present in the snowpack (see Tables 2 and 3 and Fig. 3a). Meltwaters of the second event were heavily influenced by the black snow because (1) the snow was at the surface at the onset of the second melt event, giving rise to the first meltwater, and (2) the solute content of the black snow was high and of different composition from the existing snowcover.

Figure 3b shows that meltwaters collected at site 2 seem to be a mixture of two types: those derived from the existing snowcover, proportionally enriched in chloride, and those derived from the black snow, proportionally enriched in sulphate and nitrate. The high concentrations of sulphate and nitrate (see Table 3) further suggest that the black snow contributed most of these ions, because an unrealistically large fractionation of sulphate and nitrate (with respect to chloride) into meltwater would be required for the existing snowpack to provide such high concentrations²⁻⁵. A nitrate concentration $>300 \mu\text{equiv. l}^{-1}$ was found in the most concentrated sample (a data point not included in Fig. 3b).

Streamwaters at site 2 show the influence of the sulphate- and nitrate-enriched meltwater derived from the black snow during the second event (see Fig. 3b). The streamwaters became proportionally enriched in sulphate and nitrate during the second event, and exhibited similar proportions of sulphate and nitrate to the snowmelt derived from the black snow. A small depletion in the proportion of sulphate may result from adsorption of sulphate in the soil²³⁻²⁶. Thus meltwaters derived from the black snow seem to have had a major influence on the anion composition of streamwaters at site 2 during the second melt event.

The low pH recorded during the second event at site 2 can be explained in a similar way, although ion-exchange reactions in the soil, involving protons and cations such as sodium, may complicate interpretation. Meltwaters are likely to have contributed much of the acidity in the streamwaters, because in this catchment, meltwater is a large component of streamwater during the acid flush^{9,10}, and meltwater pH were in the range of

3.7-3.2, all more acidic than the streamwaters and any soil waters collected in the catchment over two years of study (I.L.B., in preparation). Lysimeters recorded a decrease in soilwater pH from 4.50 to 4.30 during the second event, the lowest pH recorded in soilwater samples during two years of study (I.L.B., in preparation). This suggests that the acidic meltwaters promoted lower soilwater pH. At both sites, the Cl:Na, Al:Na and H:Na ratios increased during the second event. This suggests that ion

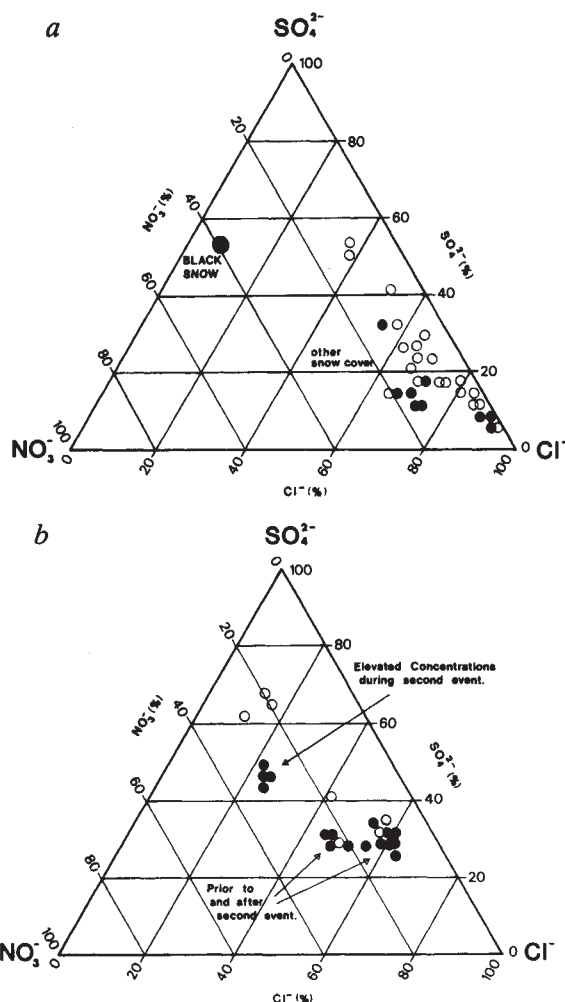


Fig. 3 a, The proportional anion composition of snows, sampled from snow pits near to site 2 on 12 January (filled circles), 8 and 9 March (open circles) and the black snowfall of 13-14 March (large filled circle). Overlying points, mainly near the chloride apex, have not been indicated. Points are plotted from data with units of $\mu\text{equiv. l}^{-1}$. b, The composition of meltwaters sampled at three depths within a snowpit, near site 2, on 15-17 March (open circles) and streamwaters at site 2, before, during and after the second event (filled circles). Overlying points have not been indicated. Points were derived from data with units of $\mu\text{equiv. l}^{-1}$.

Table 3 Concentration of anions (in $\mu\text{equiv. l}^{-1}$) and pH meltwaters (15–17 March)

pH		Cl		SO ₄		NO ₃	
Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
3.2	3.7*	37	150	44	620	19	>300

* The pH of the most dilute meltwater was not determined.

exchange reactions within the soil resulted in uptake of Na and release of Al and H. However, the available data do not allow fuller exploration of soil–meltwater interactions. Some acid must have been neutralized by the soil to give rise to the increased aluminium concentrations (Table 1), because the snowcover is a negligible source of Al (ref. 2).

If all other variables had been similar, the second acid flush should have given rise to lower ionic concentrations in the meltwaters, through to the streamwaters, because the snowpack was depleted in solute by the first melt¹⁵. This seems to be so for ions that were not major solute components of the black snow. The increase over background levels for the sea-salt component, Na⁺, Cl[−] and Mg²⁺, was 2.0, 2.5 and 2.7 respectively. This was smaller than the increase in H⁺, SO₄^{2−} and NO₃[−] (4.0, 4.3 and 7.7 respectively), which were major solute components of the black snow. Dissolved Al increased to 1/9.7 of background, with concentrations a factor of three greater than the maximum measured over the previous two years. This was probably caused by the lower streamwater pH. These arguments also suggest that the anion concentrations and pH of streamwaters during the second event were unusual and influenced by meltwaters derived from the black snowfall.

These results have implications for future research on meltwater composition and the effects of acidic snow on ecosystems. Future laboratory experiments concerned with the determination of snowmelt quality must include the effects of solute banding¹³ and consider solute scavenging by meltwater. The monitoring of snowpacks to predict the severity of the acid flush should be conducted in the knowledge that episodic snowfalls of high solute loading may affect streamwater chemistry, especially in the Scottish Highlands, where black snowfalls occur periodically and hydrological flowpaths permit rapid throughput of snowmelt to streamwaters.

Financial assistance was provided by NERC and the EEC.

Received 8 July 1987; accepted 1 March 1988.

- Johannessen, M. & Henriksen, A. *Wat. Resour. Res.* **14**, 615–619 (1978).
- Brimblecombe, P. *et al.* in *Modelling Snowmelt-induced Processes* (ed. Morris, E. M.) 283–295 (IAHS, Publ. No. 155, 1986).
- Davies, T. D. *et al.* in *Seasonal Snowcovers: Physics, Chemistry, Hydrology* NATO ASI Series C. Vol. 211, 337–392 (1987).
- Tranter, M. *et al.* *Atmos. Envir.* **20**, 517–525 (1986).
- Tranter, M. *et al.* *Wat. Air Soil Pollut.* **36**, 75–90 (1987).
- UK Review Group on Acid Rain Acid Deposition in the UK (Warren Spring Laboratory, Stevenage, 1983).
- Leivestad, H. & Muniz, I. P. *Nature* **259**, 391–392 (1976).
- Gunn, J. M. & Keller, W. *Can. J. Fish. aquat. Sci.* **41**, 319–329 (1984).
- Morris, E. M. & Thomas, A. G. *Wat. Air Soil Pollut.* **34**, 429–438 (1987).
- Cooper, D. M., Morris, E. M. & Smith, C. J. *J. Hydrol.* **93**, 221–240 (1987).
- Davies, T. D. *et al.* *Nature* **312**, 58–61 (1984).
- Davies, T. D. *et al.* in *Proc. NATO ARW Acid Deposition Processes at High Elevation Sites, Edinburgh, 1986* (in the press).
- Marsh, A. R. W. & Webb, A. H. *Physico-Chemical Aspects of Snow-Melt*, CEBG Rep. RD/L/N, 60/79 (1979).
- Romans, J. C. C. *Welsh Soils Grp Rep.* **11**, 88–101 (1970).
- Thompson, I. P., Blackwood, I. L. & Davies, T. D. *Envir. Pollut.* **43**, 143–154 (1987).
- Morris, E. M. & Thomas, A. G. *J. Glaciol.* **31**, 190–193 (1985).
- Tsiouris, S. *et al.* *Ann. Glaciol.* **7**, 196–201 (1985).
- Tsiouris, S. thesis, Univ. East Anglia (1986).
- Abrahams, P. W. *et al.* in *Proc. Annual Conf. Trace Substances in Envir. Hlth.*, Univ. Missouri, June 1985, 67–77 (University of Missouri Press, 1985).
- Abrahams, P. W. *et al.* *Wat. Air Soil Pollut.* (in the press).
- Tranter, M. *et al.* in *Seasonal Snowcovers: Physics, Chemistry, Hydrology* NATO ASI Series C, Vol. 211, 575–597 (1987).
- Davies, T. D., Vincent, C. E. & Brimblecombe, P. *Nature* **300**, 161–163 (1982).
- Choa, T. T., Harward, M. E. & Fang, S. C. *Proc. Soil Sci. Soc. Am.* **28**, 632–635 (1964).
- Johnson, D. W. & Cole, D. W. *Air Soil Pollut.* **7**, 489–495 (1977).
- Overrein, L. N., Seip, H. M. & Tollan, A. *SNSF Project*, FR 19/80 (1980).
- Christophersen, N., Wright, R. F. & Seip, H. M. *Wat. Resour. Res.* **18**, 977–996 (1982).

Seasonal variations of D/H and ¹³C/¹²C ratios of microbial methane in surface sediments

Roger A. Burke Jr*†, Christopher S. Martens‡ & William M Sackett*

* University of South Florida, Department of Marine Science, 140 7th Avenue South, St Petersburg, Florida 33701, USA

‡ Curriculum in Marine Science, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514, USA

Application of stable isotope ratios (δD and $\delta^{13}\text{C}$) to the study of the global CH₄ cycles^{1–3} requires isotopic characterization of the chief atmospheric CH₄ sources. Shallow aquatic sediments are postulated to be an important atmospheric CH₄ source⁴; however, the stable isotope composition of CH₄ produced in these systems is not well defined. Further, the relative importance of the various processes that influence CH₄ δ values is not generally predictable and probably varies from site to site. We found appreciable seasonal and spatial (probably depth-dependent) variation in the δD and $\delta^{13}\text{C}$ of gas-phase microbial CH₄ produced in the marine sediments of Cape Lookout Bight (CLB), North Carolina⁵. In light of seasonal and depth-dependent variations of CH₄ production from acetate^{6,7} and acetate concentrations⁸ in CLB sediments, our observations provide indirect evidence that the CLB CH₄ δ variation reflects variation in the relative contributions of the main CH₄ precursors (acetate and CO₂/H₂)^{9,10}. $\delta\text{D-CH}_4$ and $\delta^{13}\text{C-CH}_4$ are inversely correlated, indicating that microbial consumption^{11,12} is not responsible for the δ variation. Owing to its marine nature, CLB is not strictly representative of wetlands in general; however, some of the key microbial processes and environmental characteristics that appear largely responsible for the observed CLB CH₄ δ variation are probably similar in typical temperate and high-latitude wetlands.

CLB is a small, partially enclosed marine basin located on the Outer Banks of North Carolina. Sulphate-reduction and methanogenesis drive the anaerobic microbial decomposition of complex organic matter in the rapidly accumulating (~10 cm per yr)¹³ organic-rich (3–5% organic carbon)¹⁴ sediments of the bight interior (Station A-1)⁵. Seasonal variation in temperature, and possibly organic matter inputs from nearby back-barrier lagoons, induce variation in the rates of the microbial degradation processes that are responsible for production of CO₂ and CH₄ (ref. 14). From mid-May to mid-November when organic matter decomposition rates are highest, interstitial waters become sulphate-depleted and supersaturated with CH₄ within ~8 to 15 cm of the sediment-water interface⁵. Reduction of hydrostatic pressure associated with ebb tide (tidal range ~1 m, water depth ~8 m) triggers release of bubbles (85–99 mole% CH₄)^{5,15,16} to the troposphere through the water column during these months⁵. Martens *et al.*¹⁶ have attributed seasonal variations in the $\delta^{13}\text{C-CH}_4$ and $\delta^{13}\text{C-CO}_2$ of gas bubbles stirred from CLB sediments to seasonal changes in methanogenic and acetate-cycling pathways.

The δD and $\delta^{13}\text{C}$ of microbial CH₄ produced in CLB sediments exhibit clear seasonal trends which are nearly the inverse of one another, and a considerable small-scale variation (Fig. 1, Table 1). CH₄ collected during summer–fall months (July–November) is more D-depleted and less ¹³C-depleted than CH₄ sampled during winter–spring months (December–June). Seasonal and depth-dependent differences in the rate of CH₄ production in CLB sediments¹⁷ might be expected to contribute to these variations in CH₄ δ values; however, King *et al.*¹⁸ reported

† Present address: Geochemical and Environmental Research Group, 10 South Graham Road, Texas A&M University, Department of Oceanography, College Station, Texas 77840, USA.

Table 1 δD -CH₄ and $\delta^{13}C$ -CH₄ in sedimentary gas bubbles and sediment temperatures¹⁶ from Station A-1 (ref. 5) of Cape Lookout Bight

Date	Bottle number	δD -CH ₄ (‰)	$\delta^{13}C$ -CH ₄ (‰)	Sediment T (°C)	Date	Bottle number	δD -CH ₄ (‰)	$\delta^{13}C$ -CH ₄ (‰)	Sediment T (°C)
16 June 1983	1	-227	-63.5	23	14 June 1984	1	-234	-65.1	23
	2	-225	-63.7			2	-233	-64.1	
	3	-227	-63.8		2 July 1984	1	-270	-59.2	24
	4	-222	-63.9			2	-271	-59.3	
	5	-226	-63.8		18 July 1984	1	-251	-62.1	26
19 August 1983	1	-259	-62.0	27		2	-263	-61.3	
	2	-264	-60.5		10 August 1984	1	-277	-57.0	25
	3	-284	-56.8			2	-276	-56.0	
	4	-277	-58.9			3	-275	-57.9	
	5	-258	-62.2			4	-275	-58.3	
14 September 1983	1	-261	-61.4	28	31 August 1984	1	-278	-58.3	26
	2	-263	-61.9			2	-258	-61.2	
16 October 1983	1	-265	-60.0	23		3	-281	-57.4	
	2	-265	-60.3			4	-260	-60.7	
2 February 1984	1	-238	-64.3	7	19 September 1984	1	-276	-58.9	26
	2	-231	-64.3			2	-273	-59.0	
7 April 1984	1	-241	-63.6	14	17 November 1984	1	-250	-60.4	16
	2	-239	-63.0			2	-257	-60.5	
6 May 1984	1	-231	-64.0	18		3	-260	-61.0	
	2	-233	-63.9			4	-262	-60.4	
31 May 1984	1	-231	-68.4	23					
	2	-228	-68.3						

that the ^{13}C fractionation associated with conversion of CO₂/H₂ to CH₄ by a pure laboratory culture was independent of CH₄ production-rate variations of a factor of 100. The seasonal trends in CLB δD -CH₄ and $\delta^{13}C$ -CH₄ (Fig. 1, also ref. 16) appear to be related to corresponding seasonal variation of the relative contribution of acetate fermentation to total CH₄ production⁶, which probably results from seasonal variation of CH₄-zone acetate concentrations⁸. Sansone and Martens⁶ measured ^{14}C production from tracer quantities of ^{14}C -labelled acetate added to CH₄-zone sediments throughout the course of a year, and estimated that acetate fermentation could account for over 50% of the observed CLB summertime CH₄ flux. In contrast, measurements made from January to May indicated that CH₄ production from acetate was nearly-zero during the winter⁶. Acetate concentrations⁸ in CLB CH₄-zone sediments exhibit considerable seasonal (factor of ~15 to 20) and depth-dependent (factor of ~6) variation, with peak values occurring during the main summer bubbling period (July–September)¹⁴. Crill and Martens⁷ measured the depth distribution of ^{14}C production from tracer quantities of ^{14}C -labelled bicarbonate and acetate added to CLB sediments during summer (mid-July to late-August), and estimated that acetate was the precursor for 20–30% of the total CH₄ produced in the upper 30 cm of CLB sediments and up to 40% of the CH₄ produced in some depth intervals. An important implication of these observations (Fig. 1, refs 6, 7), and a key assumption of gas characterization models^{9,10}, is that acetate fermentation produces CH₄ that is relatively more D-depleted and less ^{13}C -depleted (also ref. 16) than CH₄ produced by CO₂ reduction. On the other hand, Balabane *et al.*¹⁹ found that pure cultures of *Methanobacterium formicum* grown in media with a δD near that of CLB pore water (~0‰) produced CH₄ by CO₂ reduction that was 100‰ more D-depleted than any CH₄ that we stirred from CLB sediments. A pure culture of *M. formicum* is certainly not representative of the microbial population in CLB sediments, however, and the H₂ concentrations used in the laboratory are 10⁴–10⁵ times greater than *in situ* concentrations in typical natural methanogenic sediments, in which CO₂ reduction is H₂-limited^{20,21}. In the radiotracer experiments, *in situ* substrate concentrations in the slurried⁶ or minimally disturbed⁷ samples of whole wet sediment were increased by 1% or less, and the natural microbial populations were present. Because the conditions of the radiotracer experiments^{6,7} more closely resembled

in situ CLB conditions, they should more accurately simulate natural processes.

Given the sampling method¹⁶ used in the present study and the large quantities of gas collected (up to ~600 ml), it is likely that not all 'replicates' sampled gas from the same sediment depth. The variation in δD -CH₄ and $\delta^{13}C$ -CH₄ observed among replicate samples (Fig. 1, Table 1) taken during summer (especially 19 August 1983 and 31 August 1984) could therefore be due to depth-dependent variation of the relative contribution of acetate to total CH₄ production⁷.

A negative linear correlation between δD -CH₄ and $\delta^{13}C$ -CH₄ (Fig. 2, $r = -0.90$, $n = 42$) provides good evidence that the δ variation is not simply due to seasonal and/or depth-dependent variation in the extent of bacterial methane oxidation, as this process would be expected to enrich the residual CH₄ in both D and ^{13}C (refs 11, 12), as indicated by line A in Fig. 2. An estimated 70–80% of the yearly flux of CH₄ from the CLB system occurs by ebullition⁵ through sedimentary 'bubble tubes' (ref. 15), thereby bypassing¹⁴ aerobic⁵ and anaerobic oxidation zones²². Further, linear to convex-up pore water CH₄ concentration profiles⁵ imply that transport processes outweigh anaerobic CH₄ oxidation at Station A-1 (ref. 14), and Martens *et al.*¹⁶ have reported that CH₄ released from CLB sediments by natural ebullition was only about 1‰ less ^{13}C -depleted than CH₄ released by stirring the sediment. δD -CH₄ and $\delta^{13}C$ -CH₄ of samples collected during summer-fall (July through November), when an estimated >90% of the total yearly CH₄ bubble flux occurs¹⁴, define a slightly different linear trend (Fig. 2, closed circles, $r = -0.87$, $n = 27$). The 31 May 1984 samples excepted, δD -CH₄ and $\delta^{13}C$ -CH₄ of the winter-spring samples (open circles, Fig. 2) are shifted considerably from the summer-fall trend. Partial oxidation of the trapped CH₄ bubbles associated with deeper penetration²² of the sulphate-reduction zone in CLB sediments during winter-spring⁸ could be responsible for this shift (line A' of Fig. 2). Alternatively, the shift could result from reduction of CO₂ that is less ^{13}C -depleted than that used during summer (line B of Fig. 2). Indeed, Martens *et al.*¹⁶ report that the CO₂ component of gas bubbles collected during winter was generally less ^{13}C -depleted (up to 7‰) than that of summer samples.

Although CLB is not a typical wetland, there are important similarities between CLB sediments and other more common wetlands sediments with regards to key microbial processes and

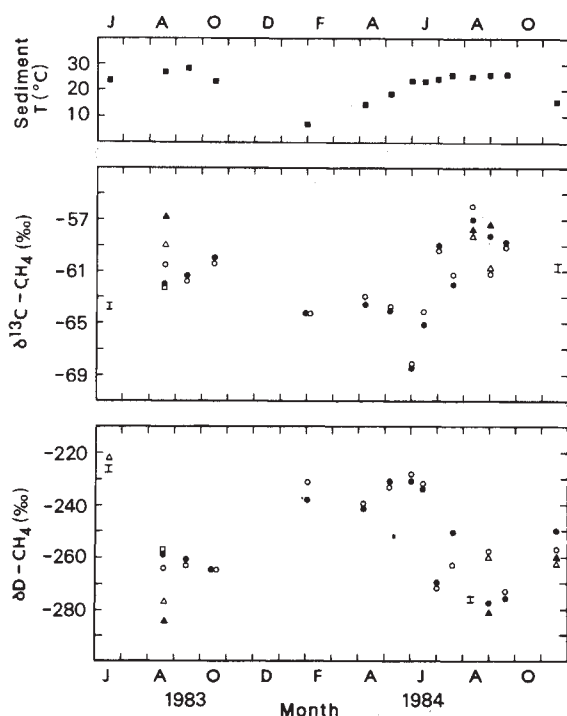


Fig. 1 $\delta D-CH_4$ and $\delta^{13}C-CH_4$ in sedimentary gas bubbles and sediment temperatures (■) from Station A-1 (ref. 5) of Cape Lookout Bight during 1983–1984. Bottle numbers: ●, 1; ○, 2; ▲, 3; △, 4; □, 5. Where data points are too closely grouped the total range is represented by $\pm$. Stable isotopic compositions are reported in the standard δ notation: $\delta(\text{‰}) = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$, where R is D/H and $^{13}C/^{12}C$, and the standards are V-SMOW (ref. 26) and PDB (ref. 27) for δD and $\delta^{13}C$ respectively. The upper 20–40 cm of the sediment were stirred by a diver to release gas bubbles into an inverted cone positioned ~1 m above the bottom. As the gas was released, it was continuously transferred from the cone to 125 ml serum bottles (except the 19 September 1984 samples (30 ml)) and stoppered at depth¹⁶. The methane component (93 to 99 mole% for this set) of the gas bubbles was prepared for isotope ratio determination with a gas chromatograph/combustion/vacuum system²⁸. The uncertainties (one standard deviation) associated with determination (2 ml CH_4) of $\delta D-CH_4$ and $\delta^{13}C-CH_4$ are 2‰ and 0.2‰ respectively²⁸.

environmental characteristics. The terminal steps of anaerobic decomposition in CLB CH_4 -zone sediments^{6–8} are similar to those operative in freshwater sediments and paddy soils²³. Further, the CLB seasonal temperature variation (Fig. 1), which appears to exert primary control on CH_4 -zone microbial processes^{8,14}, is similar in magnitude to temperature variations experienced by temperate and high-latitude wetlands, supposedly an important atmospheric CH_4 source^{24,25}. Thus the CH_4 δ relationships that we observed in CLB sediments (Figs 1 and 2) may be typical of more common wetland areas in which the dominant mode of CH_4 flux is ebullition. Indeed, seasonal variation in the $\delta^{13}C$ of CH_4 from a sub-tidal freshwater river sediment in North Carolina was observed (J. P. Chanton and C. S. Martens, personal communication) that was of similar magnitude as the CLB $\delta^{13}C$ variation (Fig. 1, ref. 16).

The seasonal (ranges: $\delta D \sim 60\%$, $\delta^{13}C \sim 12\%$) and spatial (ranges: $\delta D \sim 25\%$, $\delta^{13}C \sim 5\%$) variations that we observed support and extend the observation of Martens *et al.*¹⁶ that individual measurements may inaccurately define the stable isotopic composition of CH_4 emitted to the atmosphere from shallow aquatic environments. Agreement of the seasonal variations of CH_4 δ values (Fig. 1) with seasonal variations in CH_4 production from acetate^{6,7} is indirect evidence that acetate fermentation in CLB sediments produces CH_4 that is relatively

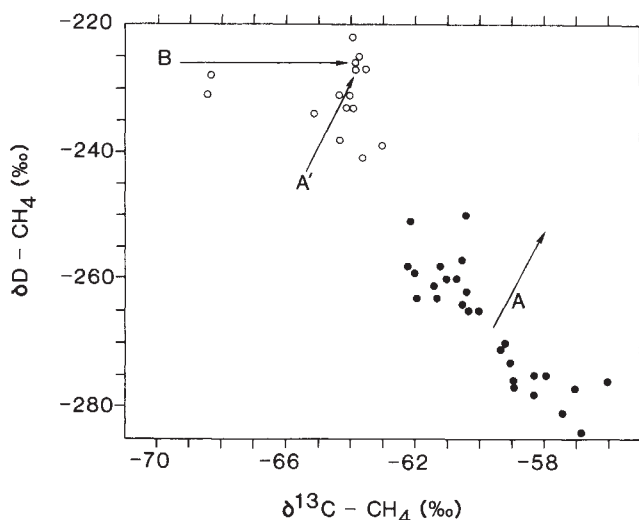


Fig. 2 $\delta D-CH_4$ vs $\delta^{13}C-CH_4$ in gas bubbles collected from Station A-1 (ref. 5) of Cape Lookout Bight during summer-fall (●) and winter-spring (○) months. The slope of lines A and A' is 10. The results of Coleman *et al.*¹¹ indicate that aerobic methane oxidation would produce a line with a slope of 8 to 14.

more D-depleted and less ^{13}C -depleted (also ref. 16) than CH_4 produced by CO_2 reduction^{9,10}. The negative linear correlation between $\delta D-CH_4$ and $\delta^{13}C-CH_4$ which we observed indicates that the isotopic variation is not simply due to CH_4 oxidation. Rather, the inverse relationship (Fig. 2) apparently represents mixing in varying proportions of CH_4 produced from acetate with CH_4 produced by CO_2 reduction, and demonstrates that simultaneous measurement of $\delta^{13}C$ and δD can provide significantly more information regarding the factors controlling CH_4 δ variation namely mixing, bacterial oxidation (line A), and substrate pool effects (line B), than can $\delta^{13}C$ measurements alone.

We thank C. Green, J. Chanton, R. Haddad, D. Burdige and other members of $CH_4\alpha O_2S$ for sample collection. E. S. Van Vleet made several suggestions that improved this manuscript. This research was supported by the NSF Marine Chemistry Program and the NASA Upper Atmosphere Research Program.

Received 3 August 1987; accepted 24 February 1988.

- Stevens, C. M. & Rust, F. E. *J. geophys. Res.* **87**, 4879–4882 (1982).
- Senum, G. I. & Gaffney, J. S. in *The Carbon Cycle and Atmospheric CO_2 : Natural Variations Archaean to Present* (eds Sundquist, E. T. & Broecker, W. S.) 61–69 (American Geophysical Union, Washington, 1985).
- Stevens, C. M. & Engelkemir, A. *J. geophys. Res.* **93**, 725–733 (1988).
- Khalil, M. A. K. & Rasmussen, R. A. *J. geophys. Res.* **88**, 5131–5144 (1983).
- Martens, C. S. & Klump, J. V. *Geochim. cosmochim. Acta* **44**, 471–490 (1980).
- Sansone, F. J. & Martens, C. S. *Science* **211**, 707–709 (1981).
- Crill, P. M. & Martens, C. S. *Geochim. cosmochim. Acta* **50**, 2089–2097 (1986).
- Sansone, F. J. & Martens, C. S. *Geochim. cosmochim. Acta* **46**, 1575–1589 (1982).
- Woltemate, I., Whiticar, M. J. & Schoell, M. *Limnol. Oceanogr.* **29**, 985–992 (1984).
- Whiticar, M. J., Faber, E. & Schoell, M. *Geochim. cosmochim. Acta* **50**, 693–709 (1986).
- Coleman, D. D., Risatti, J. B. & Schoell, M. *Geochim. cosmochim. Acta* **45**, 1033–1037 (1981).
- Alperin, M. J., Reeburgh, W. S. & Whiticar, M. J. *Global Biogeochem. Cycles* (in the press).
- Chanton, J. P., Martens, C. S. & Kipphut, G. M. *Geochim. cosmochim. Acta* **47**, 1791–1804 (1983).
- Martens, C. S. & Klump, J. V. *Geochim. cosmochim. Acta* **48**, 1987–2004 (1984).
- Martens, C. S. *Science* **192**, 998–1000 (1976).
- Martens, C. S., Blair, N. E., Green, C. D. & Des Marais, D. J. *Science* **233**, 1300–1303 (1986).
- Crill, P. M. & Martens, C. S. *Limnol. Oceanogr.* **28**, 1117–1130 (1983).
- King, S. L., Lansdown, J. M. & Quay, P. D. *Eos* **68**, 1701 (1987).
- Balabane, M., Galimov, E., Hermann, M. & Letolle, R. *Org. Geochem.* **11**, 115–119 (1987).
- Conrad, R., Phelps, T. J. & Zeikus, J. G. *Appl. envir. Microbiol.* **50**, 595–601 (1985).
- Kuivila, K. M. thesis, Univ. Washington (1986).
- Reeburgh, W. S. *Earth planet. Sci. Lett.* **47**, 345–352 (1980).
- Winfrey, M. R. in *Petroleum Microbiology* (ed. Atlas, R. M.) 153–219 (Macmillan, New York, 1984).
- Harris, R. C., Gorham, E., Sebach, D. I., Bartlett, K. B. & Flebbe, P. A. *Nature* **315**, 652–654 (1985).
- Sebach, D. I., Harris, R. C., Bartlett, K. B., Sebach, S. M. & Grice, S. S. *Tellus* **38B**, 1–10 (1986).
- Gonfiantini, R. *Nature* **271**, 534–536 (1978).
- Craig, H. *Geochim. cosmochim. Acta* **3**, 53–92 (1953).
- Burke, R. A. & Sackett, W. M. in *Organic Marine Geochemistry* (ed. Sohn, M. L.) 297–313 (American Chemical Society, Washington, 1986).

Embryonic temperature determines adult sexuality in a reptile

W. H. N. Gutzke* & David Crews†‡

* Department of Biology, Memphis State University, Memphis, Tennessee 38152, USA

† Institute of Reproductive Biology, Department of Zoology, University of Texas, Austin, Texas 78712, USA

Gonadal differentiation in amniote vertebrates is controlled by one of two mechanisms: genotypic sex determination (GSD) or environmental sex determination (ESD)¹. After differentiation the fetal gonad produces sex steroid hormones which govern the development of other components of sexuality^{2,3}. Thus, the primary sex determiner is thought to operate solely as a trigger that initiates a cascade of events culminating in adult sex differences. In the leopard gecko (*Eublepharis macularius*), gonadal and morphological sex is determined by incubation temperature, with relatively 'hot' temperatures (32 °C) resulting in mostly male offspring and relatively 'cold' temperatures (26 °C) resulting in only female offspring^{4,5}. We report here that the reproductive behaviour and endocrine physiology of an adult is influenced by the temperature experienced as an embryo. Also, the perception of a female to courtship by a male is influenced by incubation temperature. These data indicate that incubation temperature, the primary determiner of sex in this species, has differential effects on adult sexuality.

Sexual behaviour in leopard geckos is stereotyped and differs between the sexes. The typical copulatory sequence consists of the male rapidly vibrating his tail on encountering a female. He will then approach the female, licking her tail. The male next grips and shakes the female's tail; this biting and shaking is gentle and does not result in any discernible wounds. The male then shifts his grip to the female's back, neck or head area, moving his body parallel to the female's body. At this point a receptive female will remain stationary as a courting male first licks and then grips her tail. As the male shifts his grip to the female's back, neck or head area, the female arches her tail and allows intromission. A non-receptive female may break off courtship at any point by either fleeing from the male, biting the male (generally causing tissue damage) or throwing the male off her back.

The sexual behaviour of adult females from different incubation

temperatures varied significantly (Table 1; $P < 0.05$). All of the females ($n = 29$) elicited comparable levels of courtship from male stimulus animals, whereas the response of the females to male courtship differed markedly depending upon their incubation temperature. Females from cold incubation temperatures (cold females) exhibited sexual receptivity (homotypical behaviour) when courted by a male; no male-typical courtship (heterotypical) behaviours were observed in tests with either male or female stimulus animals. All of the females from hot incubation temperatures (hot females) responded frequently with heterotypical behaviours, reacting to the courtship of males as if they were themselves male. Even though hot females responded to this courtship with heterotypical behaviours, they never vibrated their tail on encountering a stimulus animal. Females from eggs incubated at 29 °C were intermediate in the frequencies of homotypical or heterotypical behaviour displayed. The behaviours of animals retested again at one and two years of age did not differ significantly from those reported here. Taken together these findings indicate that the female's perception, and not her appearance or recognition of others, has been influenced by incubation temperature. Whether perception is influenced by gonadal hormones, as is the case in mammals⁶, remains to be determined.

Over a 2-year period, none of the hot females were observed to mate or lay eggs. Histological examination of the ovaries of hot females did not reveal any testicular tissue or cells (Fig. 1). Whether this apparent sterility of hot females occurs at the level of the gonad, hypothalamus and/or pituitary remains to be determined. In mammals, administration of androgen to neonatal females results in an anovular (sterile) adult⁷. Males from different incubation temperatures did not differ significantly in their homotypical or heterotypical behaviour to male or female stimulus animals (Table 1). However, males were produced over a smaller range of temperature (3° versus 6 °C) and this may be why less behavioural variation was observed.

Agonistic behaviour is equally stereotyped but sexually dimorphic only in frequency, not in form. Agonistic behaviour in both sexes was affected by incubation temperatures ($P < 0.05$ for comparison of treatment groups within a sex) (Fig. 2). Both males and females were more likely to exhibit aggressive behaviour if they had experienced high temperatures during incubation; this relation was found whether animals were tested in their home cage or in a neutral site. Incubation temperature also affected the probability of aggressive behaviour by males toward other males, but not toward females. Hot females were equally aggressive toward either sex.

Table 1 Effect of incubation temperature on psychosexual differentiation in the leopard gecko, *Eublepharis macularius*

Experimental animal	Female				Male			
	26 °C		29 °C		32 °C		32 °C	
Incubation temperature	12		11		6		10	
Sample size								
Stimulus animal	M	F	M	F	M	F	M	F
Number showing only homotypic (or expected response)	12	12	2	10	0	4	9	10
Number showing only heterotypic response	0	0	1	0	5	0	0	0
Number showing both responses	0	0	8	1	1	2	1	0

$P < 0.05$

In 1985, experimental animals were housed individually in plastic cages for at least 14 days before testing. In 1986, experimental animals were tested both in their cages and in newly washed cages. The stimulus animals were introduced into the experimental animal's cage at the time of testing using a 'round robin' design. Because leopard geckos are crepuscular, all tests were performed between 16:00 (1 h before lights off) and 20:00 hours. Individuals were tested for sexual and agonistic behaviours upon attaining a body mass of 25.0 g. Experimental animals were scored on the basis of their behavioural responses to each stimulus animal. Five males and five females known to have reproduced were selected as stimulus animals; the incubation history of these individuals was not known as they were acquired from other laboratories. Each experimental animal was tested with each stimulus animal once during the course of the experiment. The sex of the stimulus animal was varied on alternate nights; half of the animals from each experimental group were tested for their response to a male stimulus animal on the first day whereas the other half were tested for their response to a female stimulus animal. Because copulation affects both receptivity and attractivity in some reptiles^{17,18}, mating was not permitted. Behavioural tests lasted for 15 min or until a definitive response (for example, attempted copulation or attack) was noted. In 1985 individuals were tested once whereas in 1986 animals were tested three times; at 6 months, at 1 year and at 2 years of age.

‡ To whom correspondence should be addressed.

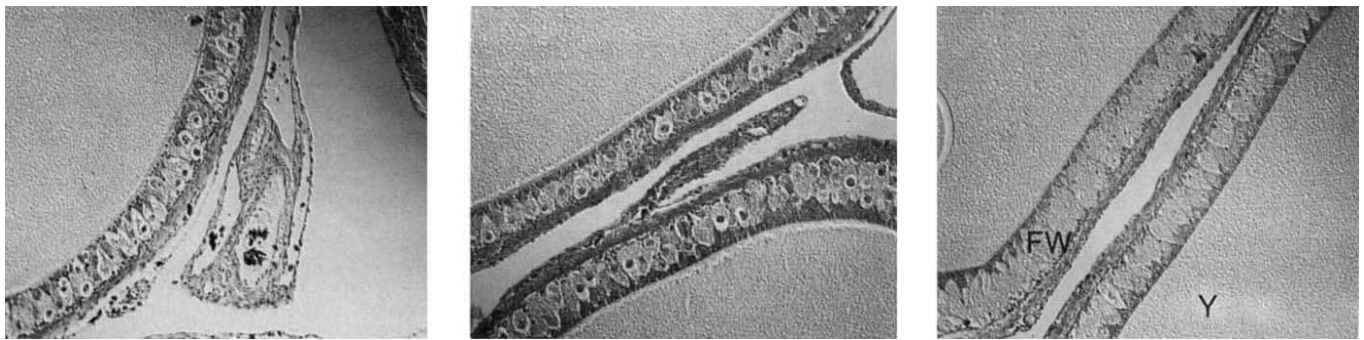


Fig. 1 Histology of ovaries of adult leopard geckos that had been incubated at 26 (left), 29 (centre) and 32 °C (right), as viewed by Normarski differential interface contrast optics. Magnification 35 diameters. Y, yolk; FW, follicle wall. Tissues were imbedded in paraffin, cut at 6 μ m, and stained with trichrome series. Eggs were incubated in covered plastic cups containing sterilized vermiculite at 26, 29 or 32 °C (± 1.0 °C) in 1985 and in 1986. The water potential of the substrate was maintained near saturation in all treatment groups throughout incubation because water stress is known to affect sex determination in other reptiles that exhibit temperature-dependent sex determination^{20,21}. At emergence, each hatchling was individually numbered and marked for future identification. All animals were reared under controlled conditions (~ 27 °C; 14-h photoperiod). In 1985, gonadal sex was determined by laparotomy and gonadal inspection within one month after hatching and then again at 6–8 months; at this time males produce motile sperm but females have not yet achieved sexual maturation². In 1986, the sex of individuals was assigned according to the presence of secondary sex characters and later confirmed at autopsy. Normally, gonadal males are identified by enlarged scales and swelling near the venter whereas gonadal females lack these secondary characters. In 1985, hatchlings were raised in mixed sex groups of 10–20 individuals, whereas in 1986, hatchlings were raised in isolation. Housing conditions affected the time to sexual maturity but not the pattern of responses once maturity was reached; individuals in isolation required 1–2 months longer to become sexually active and had smaller gonads than individuals raised in community boxes.

Incubation temperature of the embryo affected other sexual dimorphisms in adult phenotype. The endocrine physiology of the adult was influenced by the temperature experienced during incubation. Radioimmunoassay of circulating concentrations of sex steroid hormones revealed systematic differences in the levels of total androgens and oestradiol both between and within the sexes (Table 2). Androgen levels were highest and oestrogen

Table 2 Circulating concentrations of total androgens and estradiol as measured by radioimmunoassay in adult leopard geckos (*Eublepharis macularius*)

Incubation temperature	Number	Total androgens	Oestradiol
Female			
26	4	0.36 (0.07)	0.88 (0.13)
29	7	1.23 (0.45)	0.49 (0.05)
32	5	6.06 (2.11)	0.41 (0.10)
Male			
29	6	77.92 (26.40)	0.48 (0.06)
32	9	31.67 (4.33)	0.37 (0.04)

Mean concentration in ng ml⁻¹ is presented with ± 1 standard error in parentheses. Blood samples were taken from the postorbital sinus at the end of the behavioural testing from animals in both years and circulating concentrations of total androgens and oestradiol determined by radioimmunoassay using methods described in ref. 19. These data were subjected to two-way analysis of variance (ANOVA) with incubation temperature and sex as fixed factors.

levels lowest in adult females that had experienced relatively hot incubation temperatures ($P < 0.05$). Also, behavioural differences among individuals within the same treatment group reflected these hormonal differences. For example, one female incubated at 32 °C exhibited a much lower frequency of heterotypic behaviour compared with other females incubated at this temperature; this female also had the lowest level of androgen and the highest level of oestrogen compared with the other hot females. Overall, circulating concentrations of total androgens in adult females were lower than in adult males ($P < 0.05$); levels of oestrogen did not differ significantly between the sexes. No significant differences were detected in the hormonal profiles between males from eggs incubated at different temperatures.

Three lines of evidence indicate that differential effects of the

environment on development also occur in nature. First, other species which exhibit ESD in the laboratory also exhibit it in nature^{8,9}. Second, environmental conditions in natural nests cause phenotypic variation in a suite of hatchling and juvenile characteristics in other oviparous reptiles¹⁰. Finally, in a nest-selection experiment with leopard geckos, females offered a thermal gradient which reflected the range encountered in nature (based on climatological data of Iran and western Pakistan) selected a range of nest temperatures which encompass those used in this study¹¹. Thus, our findings provide support for the observation that variability among adults in a diverse suite of phenotypic characteristics in species exhibiting ESD, including sexual behaviours, need not be the result of genetic differences among individuals but may be caused by environmental differences encountered during embryonic development¹².

The long term effects of temperature experienced during incubation in the leopard gecko may be similar to the effects of perinatal hormones in mammals. Research with mammals that produce large litters (polytocous reproduction) indicates that variation in sexual dimorphisms are related to the hormonal environment experienced during development^{13–15}. In these species, the hormonal milieu of a fetus varies with the individual's intrauterine position: females developing between two male fetuses (2M females) have higher amniotic fluid and blood titers of androgen, and lower levels of oestrogen, than do female fetuses that do not develop next to a male (0M females)¹³. Females also exhibit significant differences in their morphology, physiology, and reproductive behaviour: (1) compared to 0M females, the genitalia of 2M females resemble more closely that of males; (2) 2M females are slower to reach puberty and have shorter and fewer reproductive cycles as adults; and (3) 2M females are masculinized in their sexual and aggressive behaviour, being less attractive and sexually arousing to males and more aggressive.

These results also have implications for theories involving the evolution of sex determining mechanisms^{1,16} because the primary determinant, in this case temperature, also directly affects non-gonadal aspects of sexuality. The present study indicates that the reproductive success (fitness) of adults in ESD species may be influenced by the temperature experienced during embryogeny. One consequence of temperature-dependent sex determination in leopard geckos is that 'hot' females, or as many as 20% of the individuals experiencing high incubation

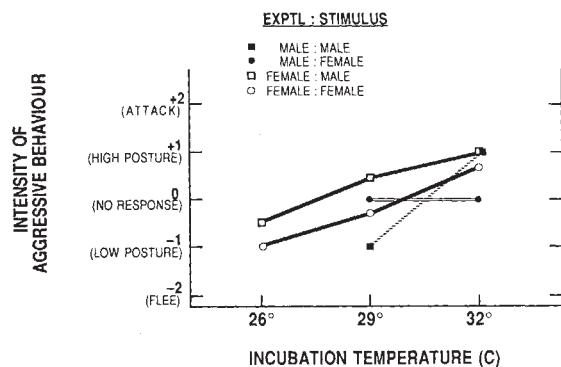


Fig. 2 Effect of incubation temperature on agonistic behaviour in the leopard gecko, *Eublepharis macularius*, for various combinations of male/female as experimental subject or stimulus. Presented are the average median behavioural responses of adult geckos in tests with both male and female stimulus animals. Sample sizes are as in Table 1. Animals may flee from the approach of another individual while maintaining a low body posture and exhibiting rapid tail twitches. Submissive behaviour is also characterized by the individual remaining stationary but assuming a splayed body posture when approached. But an individual may not alter its normal posture when confronted by an approaching animal. There are two levels of aggressive behaviour. Low-level aggression is characterized by the individual fully extending its limbs, arching its back, and raising its tail in response to an intruder. Intense aggression is characterized by the animal assuming a high body posture and attacking and biting the other animal; this response often results in tissue damage to the individual being attacked.

temperatures may be functionally sterile. This finding is consistent with theoretical considerations regarding the evolution of ESD¹⁶.

We have shown that in the leopard gecko incubation temperature, which determines gonadal sex in this species, also profoundly affects the reproductive behaviour and physiology of the adult. This discovery provides an animal model system with which to investigate the effects of the environment on various levels (morphology, physiology, and behaviour) of sexual dimorphisms in amniote vertebrates. It also suggests questions regarding the constraints of ESD not encountered in GSD.

We thank J. J. Bull for the leopard geckos, J. J. Bull and M. Mendonça for discussion, and S. Gutzke, Y. Morris, A. Alexander and J. LaClaire for technical assistance. This work was supported in part by a National Institute of Mental Health Research Development Award to D. Crews and an NSF grant to J. J. Bull.

Received 16 December 1987; accepted 18 March 1988.

1. Bull, J. J. *Evolution of Sex Determining Mechanisms* (Cummings, Menlo Park, 1983).
2. Wilson, J. D., George, F. W. & Griffin, J. E. *Science* **211**, 1278-1284 (1981).
3. Crews, D. in *Masculinity/Femininity: Basic Perspectives* (eds Reinisch, J. M., Rosenblum, L. A. & Sanders, S. A.), 83-106 (Oxford University Press, 1987).
4. Wagner, E. Q. *Rev. Biol.* **55**, 21 (1980).
5. Bull, J. J. *J. exp. Zool.* **241**, 143-148 (1987).
6. Beach, F. A. *Can. J. Psychol.* **37**, 193-210 (1983).
7. Goy, R. W. & McEwen, B. S. *Sexual Differentiation of the Brain* (MIT Press, Cambridge, Massachusetts 1980).
8. Bull, J. J. *Ecology* **66**, 1115-1122 (1985).
9. Spotila, J. R., Standora, E. A., Morreale, S. J., Ruiz, G. J. *Herpetologica* **43**, 74-79 (1987).
10. Packard, G. C., Paukstis, G. L., Boardman, T. J. & Gutzke, W. H. N. *Can. J. Zool.* **63**, 2422-2429 (1985).
11. Bull, J. J., Gutzke, W. H. N. & Bulmer, M. G. *J. evol. Biol.* (in the press).
12. Pittendrigh, C. S. in *Behaviour and Evolution* (eds Roe, A. & Simpson, G. G.) 390-416 (Yale University Press, New Haven, 1958).
13. VomSaal, F. in *Hormones and Behaviour in Higher Vertebrates* (eds Balthazart, J., Prove, E. & Gilles, R.) 158-177 (Springer, Berlin, 1983).
14. Ward, I. L. & Ward, O. B. *Handbook of Behavioural Neurobiology*, Vol. 7, *Reproduction* (eds Adler, M., Pfaff, D. W. & Goy, R. W.) 77-100 (Plenum, New York, 1985).
15. Clarke, M. M. & Galef, B. G. *Physiol. Behav.* (in the press).
16. Charnov, E. L. & Bull, J. *Nature* **266**, 828-830 (1977).
17. Crews, D. *Physiol. Behav.* **11**, 463-xxx (1973).
18. Whittier, J. M. & Crews, D. *Endocrinology* **119**, 787-792 (1986).
19. Moore, M. C., Whittier, J. M. & Crews, D. *Gen. comp. Endocr.* **60**, 144-153 (1985).
20. Gutzke, W. H. N. & Paukstis, G. L. *J. exp. Zool.* **226**, 467-469 (1983).
21. Paukstis, G. L., Gutzke, W. H. N. & Packard, G. C. *Can. J. Zool.* **62**, 1491-1494 (1984).

Retinal astrocytes are immigrants from the optic nerve

Takashi Watanabe & Martin C. Raff

Medical Research Council Developmental Neurobiology Programme, Department of Biology, Medawar Building, University College London, London WC1E 6BT, UK

The retina in most mammals contains two types of macroglial cells—Müller cells, which span the entire thickness of the retina, and astrocytes, which are mainly confined to the nerve fibre layer¹⁻⁶. Whereas Müller cells are diffusely distributed in all vertebrate retinæ, the presence and distribution of retinal astrocytes correlate with the presence and distribution of retinal blood vessels: retinæ that are avascular contain no astrocytes^{7,8}; those that are diffusely vascularized contain diffusely distributed astrocytes⁵⁻¹²; and those that are vascularized in a restricted region contain astrocytes only in the vascularized region^{7,8,13}. This striking correlation between vascularization and the presence of astrocytes led Stone and Dreher to postulate that retinal astrocytes are immigrants that enter the retina with its vasculature⁷, although others have suggested that they derive from Müller cells¹⁴. Here we provide strong evidence that astrocytes in the diffusely vascularized rat retina are immigrants from the optic nerve.

Whole mounts of retina from perinatal Sprague-Dawley rats were stained with antibodies against glial fibrillary acidic protein (GFAP) to identify astrocytes¹⁵ (see ref. 16 for a review of the evidence that GFAP can be used as an astrocyte-specific marker in the mammalian retina). GFAP⁺ cells were first seen between embryonic day 18 (E18) and E19 and they were largely confined to the optic nerve head. At E21 GFAP⁺ cells were also seen in the retina, but only out to a distance of ~800 µm from the edge of the nerve head. By postnatal day 3 (P3), GFAP⁺ cells were seen as far as 1.7 mm from the edge of the nerve head and by P9 some had reached the periphery of the retina (Fig. 1). Similar results have been obtained previously in the developing rat^{12,17} and cat¹⁸ retina. These findings are consistent with the view that astrocytes migrate centrifugally from the optic nerve head to the rest of the retina, but they could also be explained by a centrifugal gradient of astrocyte differentiation across the retina.

To help distinguish between these two possibilities we studied the ability of neuroepithelial cells from different parts of the retina to generate GFAP⁺ astrocytes in culture. We showed previously that astrocytes develop at the same time as they would *in vivo* in dissociated cell cultures of developing rat brain¹⁹ and optic nerve²⁰. When cells from E14 to E18 retina (including the optic nerve head) were cultured under the conditions described previously for brain cell cultures¹⁹, GFAP⁺ astrocytes always developed at the expected time, the equivalent of E18 to E19 (not shown). In contrast, when cells from the peripheral half or two thirds of E18 retina were cultured for up to 10 days under the same conditions, no GFAP⁺ cells developed, even though a total of more than 10⁵ cells were scanned in each culture; as a control, cells from the central part (including the optic nerve head) of the same retinae were cultured, and GFAP⁺ astrocytes always developed within a few days (Fig. 2 and Table 1). As reported previously for cultures of neonatal rat retina²¹, all of the GFAP⁺ cells had the antigenic phenotype of type-1 astrocytes²²; they were labelled by the anti-Ran-2 monoclonal antibody²³, but not by the A2B5 monoclonal antibody²⁴.

As shown in Table 1, GFAP⁺ astrocytes also failed to develop in explant cultures of outer E18 retina although they did develop in explant cultures of inner E18 retina containing the optic nerve head. In the latter case, immunofluorescence staining of the explanted tissue revealed GFAP⁺ astrocytes extending ~1 mm from the edge of the nerve head after 8 days in culture (not shown).

Fig. 1 The spread of GFAP⁺ astrocytes in the developing rat retina. The distances from the edge of the optic nerve head to the peripheral edge of the retina (○) and to the most peripheral GFAP⁺ cells (*) are plotted against developmental age. The rats were usually born at E21.

Methods. Neural retinae of perinatal Sprague-Dawley rats were dissected as described²¹. The whole retina was immersion-fixed in 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 (2% PFA) for 2 h at room temperature, washed in half-strength phosphate buffered saline (PBS) with saponin (1.5 µg ml⁻¹) and 2% fetal calf serum (FCS) for 2 h at room temperature, and then floated in rabbit anti-GFAP antiserum³⁶ (diluted 1:100 in the same buffer) for 2 days at 4 °C. After a wash in the same buffer for 2 h, the retina was floated in donkey anti-rabbit immunoglobulin coupled to biotin (Amersham, diluted 1:50 in the same buffer) for 2 days at 4 °C. After another wash for 2 h in the same buffer, the retina was floated in fluorescein-coupled streptavidin (Amersham, diluted 1:100 in the same buffer) for 2 days at 4 °C. All of the incubations were done on a gently rocking platform. After the final wash, the retina was spread onto a gelatinized glass slide, mounted in glycerol containing 1,4-diazobicyclo(2,2,2)octane to inhibit fading of the fluorescence, and then examined in a Zeiss Universal microscope. The distances from the edge of the optic nerve head to the edge of the retina and to the most peripheral GFAP⁺ cells were measured with a micrometer in one eyepiece. Each point is the mean $\pm$ s.d. of measurements made on at least 10 retinae obtained from at least three rat litters.

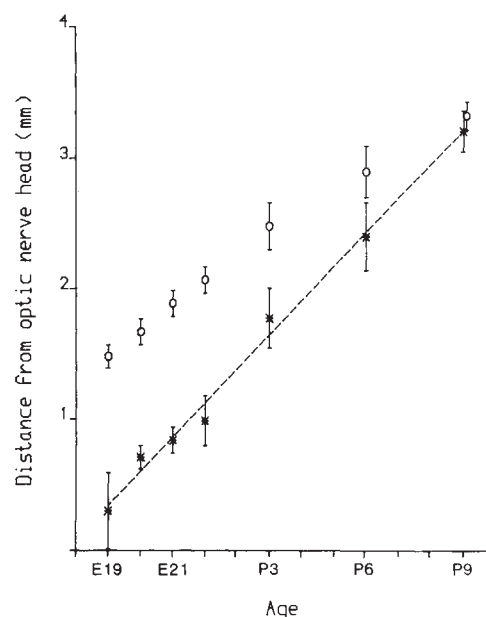
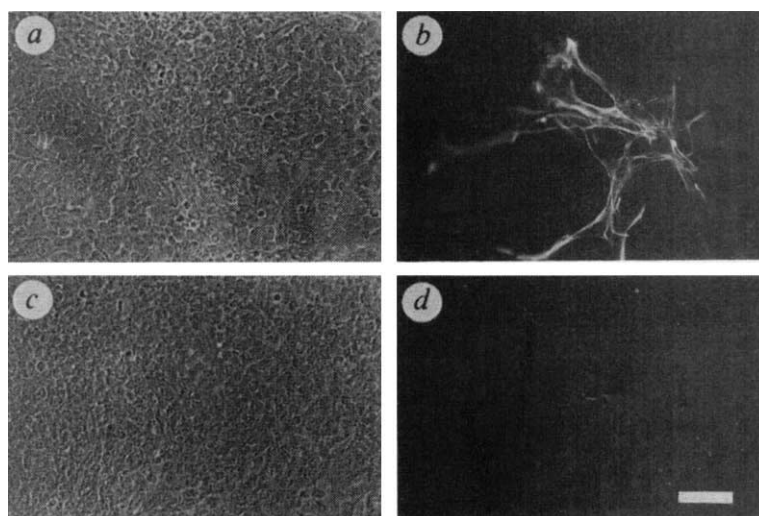


Fig. 2 Immunofluorescence (b and d) and phase contrast (a and c) micrographs of cells cultured from central (a and b) and peripheral (c and d) halves of E18 retina. After 8 days *in vitro* the cells were stained with anti-GFAP antiserum. GFAP⁺ astrocytes developed only in cultures of cells prepared from the central half of the retina, which included the optic nerve head (a and b). Scale bar, 25 µm.

Methods. Neural retinae were dissected from E18 Sprague-Dawley rats. The optic nerves were cut off just behind the optic nerve head. The neural retina was divided into two parts by cutting circumferentially around the retina approximately half way between the centre and the peripheral edge of the retina to give a central and a peripheral part. Each part was incubated for 10 min at 37 °C in 2 ml of Ca²⁺- and Mg²⁺-free minimum Eagle's medium (MEM) with 0.02 M HEPES buffer (Ca²⁺, Mg²⁺-free MEM-HEPES) containing 0.125% (w/v) trypsin (Sigma). The tissue was then transferred to 2 ml of Ca²⁺-, Mg²⁺-free MEM-HEPES containing DNase (Sigma, 0.04 mg ml⁻¹), soybean trypsin inhibitor (Sigma, 0.52 mg ml⁻¹) and bovine serum albumin (Sigma, 3 mg ml⁻¹) and dissociated by gentle trituration through a Pasteur pipette. After washing the cells with MEM containing 10% FCS, 200,000 cells were suspended in 40 µl of Dulbecco's modified Eagle's medium supplemented with 10% FCS (DMEM-FCS) and plated on poly-L-lysine (PLL)-coated coverslips in 24-well tissue culture plates (Falcon). After incubating for 30–60 min at 37 °C to allow the cells to adhere, 500 µl of the same medium was added and the cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air for 8 days at 37 °C. The cells were fixed in 2% PFA for 5 min at room temperature followed by acid-alcohol for 10 min at -20 °C and then labelled with anti-GFAP antiserum (diluted 1:300) followed by fluorescein-conjugated sheep anti-rabbit immunoglobulin (Sh anti-Rlg-Fl; Wellcome, diluted 1:100). The coverslips were mounted as described in Fig. 1 legend, and photographed with Tri-X film rated 400 ASA.



These results suggest either that retinal astrocytes (or their precursors) migrate into the developing retina from the optic nerve head, or that retinal neuroepithelial cells only differentiate into astrocytes if they are signalled by another cell type that is present in the central, but not in the peripheral, part of the E18 retina; mesenchymal cells, for example, which have been shown to migrate into the retina from the optic nerve head^{25,26} might be required for astrocyte differentiation. To help distinguish between these two possibilities central and peripheral E18 retinal cells were mixed together and co-cultured, after proliferating cells of one of the two populations had been prelabelled with bromodeoxyuridine (BrdU) (the BrdU is incorporated into replicating DNA and can be detected with anti-BrdU antibody²⁷). After 5 or 7 days the co-cultures were double-labelled with anti-BrdU and anti-GFAP antibodies. When the central retinal cells were prelabelled with BrdU, ~30–40% of the GFAP⁺ cells that developed in the co-cultures were BrdU⁺, whereas when the peripheral cells were prelabelled with BrdU, none of the

GFAP⁺ cells were BrdU⁺, suggesting that astrocytes only developed from central retinal cells in these co-cultures (Table 2). BrdU treatment did not affect the number of GFAP⁺ cells that developed in retinal cell cultures or the time that GFAP⁺ cells first appeared *in vivo* or *in vitro*. As central retinal cells were present in these co-cultures these findings make it very unlikely that the failure of neuroepithelial cells from the peripheral E18 retina to generate astrocytes in culture is due to a requirement for signals from a cell-type that is present only in the central retina or optic nerve head.

Our results thus strongly suggest that astrocytes do not develop from retinal neuroepithelial cells, but, instead, type-1 astrocytes and/or their precursor cells migrate into the developing retina from the optic nerve head, at a rate of ~265 µm per day (~11 µm h⁻¹) in the rat (Fig. 1). It remains to be determined how the close relationship between the astrocytes and blood vessels arises^{7,28,29}. Although astrocytes and/or their precursors have been shown to migrate from CNS grafts into host CNS tissue^{30,31},

Table 1 Development of GFAP⁺ astrocytes in perinatal rat retina and in culture of E18 rat retina

Part of retina	Total GFAP ⁺ cells and (total retinal cells × 10 ⁻⁵)			
Development <i>in vivo</i>	Age			
	E18	E21	P3	P7
Central retina	0 (4.3 ± 0.5)	1,308 ± 166 (18.5 ± 12.2)	5,903 ± 480 (85.0 ± 5.0)	9,282 ± 457 (85.0 ± 5.0)
Peripheral retina	0 (7.6 ± 0.6)	0 (31.1 ± 3.0)	629 ± 46 (58.7 ± 3.1)	3,231 ± 404 (104.0 ± 10.8)
Development <i>in vitro</i>	Days in culture (E18+)			
	0	3	6	10
Dissociated cell culture:				
Central retina	0	371 ± 68 (3.8 ± 0.4)	941 ± 53 (7.3 ± 0.4)	1,185 ± 323 (6.3 ± 1.1)
Peripheral retina	0	0 (6.3 ± 0.7)	0 (16.6 ± 3.1)	0 (18.1 ± 2.0)
Explant culture:				
Central retina	0	ND	614 ± 95 (25.4 ± 1.2)	833 ± 74 (17.8 ± 0.7)
Peripheral retina	0	ND	0 (32.7 ± 4.2)	0 (26.9 ± 3.5)

To study astrocyte development *in vivo*, neural retinae from E18, E21, P3 and P7 Sprague-Dawley rats were divided into central and peripheral parts and dissociated into single cells as described in Fig. 2 legend. Total numbers of cells from each part of the retina at each age were counted in a haemocytometer and 100,000 cells were plated on PLL-coated coverslips and cultured for 4 h in DMEM-FCS before fixing and staining as in Fig. 2. To study astrocyte development in dissociated cell culture, E18 rat retina cells were prepared and cultured as described in Fig. 2 legend. After 3, 6 and 10 days in culture (equivalent to E21, P3 and P7 *in vivo*), cells were removed from the coverslips by treating with 0.02% (w/v) EDTA in Tris-buffered saline (EDTA) containing 0.125% (w/v) trypsin for 10 min. After washing the cells in MEM containing 10% FCS, the total number of cells from each coverslip was counted, and 100,000 cells were plated on PLL-coated coverslips for 4 h before fixing and staining. To study astrocyte development in explant culture, E18 neural retinae were divided into two parts as described in Fig. 2 and pieces of central and peripheral retina were cultured separately on autoclaved polycarbonate filters (Nucleopore Corp) floating in DMEM-FCS. After 6 and 10 days in culture, the explants were removed from the filters and dissociated into a single cell suspension as described in Fig. 2 legend. After counting the total number of cells derived from each part of retina, 100,000 cells were plated on PLL-coated coverslips for 4 h before fixing and staining. The total number of GFAP⁺ astrocytes in each half-retina or the total that developed in culture from each half-retina, was calculated by multiplying the percentage of GFAP⁺ cells by the total number of cells (shown in parentheses) obtained from each half-retina. The results are expressed as means ± s.d. of at least three separate experiments. ND = not done.

to our knowledge they have not previously been shown to migrate during normal CNS development. Our evidence that type-1 astrocytes and/or their precursors migrate into the developing rat retina raises the possibility that they may also migrate in other regions of the developing CNS. The glial progenitor cells that give rise to both oligodendrocytes and type-2 astrocytes³² also seem to migrate long distances within the CNS^{33,34}.

Our results indicating that retinal neuroepithelial cells do not give rise to astrocytes suggest a probable explanation for a recent finding of Turner and Cepko³⁵, who used retrovirus-mediated gene transfer to study cell lineages in the neonatal rat retina. Although they found virus-labelled clones of cells containing various combinations of neuronal cell types and Müller cells, they did not find any labelled clones containing astrocytes. It seems that early in development the retinal neuroepithelium becomes committed to generating only one type of glial cell (Müller cells) as well as the major classes of retinal neurons, in

Table 2 Development of GFAP⁺ astrocytes in mixed cultures of central and peripheral E18 retina cells

Cells cultured	Cells prelabelled with BrdU	Number of GFAP ⁺ cells	Number of GFAP ⁺ , BrdU ⁺ cells
Experiment 1			
Central	Central	921 ± 116	396 ± 61
Peripheral	Peripheral	0	0
Central + peripheral	Central	520 ± 163	223 ± 72
Central + peripheral	Peripheral	526 ± 96	0
Experiment 2			
Central	Central	473 ± 54	149 ± 26
Peripheral	Peripheral	0	0
Central + peripheral	Central	223 ± 61	68 ± 20
Central + peripheral	Peripheral	212 ± 44	0

Pregnant Sprague-Dawley rats at 17 days gestation were given three intraperitoneal injections, 12 h apart, of bromodeoxyuridine (BrdU, Boehringer; 40 mg in 2 ml of MEM-HEPES). Two hours after the last injection, the rats were killed and cells were prepared separately from peripheral and central parts of the embryonic retinae as described in Fig. 2. In each experiment cells from BrdU-labelled rats and from unlabelled E18 rats were mixed together (100,000 cells from each) and cultured as described in Fig. 2 to form either all-central or all-peripheral cell cultures, or mixed central and peripheral cell cultures. In experiment 1, cells were prepared from the peripheral and central thirds of the retina, while in experiment 2 they were prepared from the peripheral and central halves of the retina. After 5 days in experiment 1 and 7 days in experiment 2, the cells were removed from the culture dish, cultured on coverslips for 4 h, fixed in 70% ethanol for 30 min at -20 °C, and then labelled with anti-GFAP serum followed by Sh anti-R1G-F1 as described in Table 1 and Fig. 2. The cells were then treated for 10 min at room temperature first with 2 N HCl (to denature the nuclear DNA³⁷), then with 0.1 M Na₂B₄O₇, pH 8.5, before labelling with monoclonal anti-BrdU antibody³⁸ (culture supernatant diluted 1:5) followed by goat anti-mouse immunoglobulin antibody coupled to rhodamine (Cappel, diluted 1:50 in 1% Triton X-100 and 2% FCS in PBS). Of the retinal cells freshly dissociated from BrdU-labelled rats ~70% were stained with anti-BrdU antibody, while ~40 and 30% were stained in unmix cultures of such cells after 5 and 7 days, respectively. The results shown are the means ± s.d. of three experiments (experiment 1) or of triplicates in a single experiment (experiment 2).

the same way that the optic stalk neuroepithelium seems to become committed to generating only type-1 astrocytes³⁴. The factors that dictate such region-specific commitments within the continuous neuroepithelium remain to be discovered.

We thank David Mason for anti-BrdU antibody, our colleagues for helpful comments on the manuscript, Anne Mudge for an important suggestion and Julia Burne for help with photography. T.W. is supported by a grant from the British Council.

Received 11 February; accepted 18 March 1988.

- Marchesani, O. & von Graefes *Arch. clin. exp. Ophthalmol.* **117**, 575-605 (1926).
- Wolter, J. R. in *The Structure of the Eye* (ed. Smelser, A. K.) 117-138 (Academic, New York, 1961).
- Lessell, S. & Kuwabara, T. *Arch. Ophthalmol.* **70**, 671-678 (1963).
- Ramon, y. Cajal, S. *The Structure of the Retina* (Charles C. Thomas, Springfield, 1972).
- Büssow, H. *Cell Tissue Res.* **206**, 367-378 (1980).
- Ogden, T. E. *Invest. Ophthalmol. vis. Sci.* **17**, 499-510 (1978).
- Stone, J. & Dreher, Z. *J. comp. Neurol.* **255**, 35-49 (1987).
- Schnitzer, J. *Glia* **1**, 74-89 (1988).
- Karschin, A., Wässle, H. & Schnitzer, J. *J. comp. Neurol.* **249**, 564-576 (1986).
- Shaw, G. & Weber, K. *Eur. J. cell Biol.* **33**, 95-104 (1984).
- Ikui, H., Uga, S. & Kohno, T. *Ophthalm. Res.* **8**, 100-110 (1976).
- Dixon, R. G., & Eng, L. F. *J. comp. Neurol.* **195**, 305-321 (1981).
- Schnitzer, J. *J. comp. Neurol.* **240**, 128-142 (1985).
- Reichenbach, A. & Wohlrab, F. *J. Neurocytol.* **15**, 451-459 (1986).
- Bignami, A., Eng, L. F., Dahl, D. & Uyeda, C. T. *Brain Res.* **43**, 429-435 (1972).
- Schnitzer, J. in *Progress in Retinal Research Vol. 7* (eds Osborne, N. & Chader, J.) 209-231 (Pergamon, Oxford, 1987).

17. Ling, T., Mitrofanis, J. & Stone, J. *Neurosci. Lett. Suppl.* **28**, S92 (1988).
18. Ling, T. & Stone, J. *Neurosci. Lett. Suppl.* **27**, S96 (1987).
19. Abney, E. R., Bartlett, P. P. & Raff, M. C. *Dev. Biol.* **83**, 301-310 (1981).
20. Raff, M. C., Abney, E. R. & Miller, R. H. *Dev. Biol.* **106**, 53-60 (1984).
21. French-Constant, C., Miller, R. H., Burne, J. F. & Raff, M. C. *J. Neurocytol.* (in the press).
22. Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R. & Noble, M. J. *Neurosci.* **3**, 1289-1300 (1983).
23. Bartlett, P. F. *et al. Brain Res.* **204**, 339-351 (1981).
24. Eisenbarth, G. S., Walsh, F. S. & Nirenberg, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4913-4917 (1979).
25. Ashton, N. *Br. J. Ophthalmol.* **38**, 385-396 (1954).
26. Henkind, P. & Oliveira, L. *Invest. Ophthalmol.* **6**, 520-530 (1967).
27. Gratzner, H. G. *Science* **218**, 474-475 (1982).
28. Janzer, R. C. & Raff, M. C. *Nature* **325**, 253-257 (1987).
29. Schnitzer, J. J. *Neurocytol.* (in the press).
30. Lindsay, R. M. & Raisman, G. *Neuroscience* **12**, 513-530 (1984).
31. Jacque, C. M., Suard, I. M., Collins, V. P. & Raoul, M. M. *Dev. Neurosci.* **8**, 142-149 (1986).
32. Raff, M. C., Miller, R. H. & Nobel, M. *Nature* **303**, 390-396 (1983).
33. Lachapelle, F. *et al. Dev. Neurosci.* **6**, 325-334 (1984).
34. Small, R. K., Riddle, P. & Nobel, M. *Nature* **328**, 155-157 (1987).
35. Turner, D. L. & Cepko, C. L. *Nature* **328**, 131-136 (1987).
36. Pruss, R. *Nature* **280**, 688-690 (1979).
37. Yong, V. W. & Kim, S. U. *J. Neurosci. Meth.* **21**, 9-16 (1987).
38. Magaud, J. P. & Mason, D. Y. *J. Immunol. Meth.* (in the press).

Cloning of a probable potassium channel gene from mouse brain

Bruce L Tempel*, Yuh Nung Jan & Lily Y. Jan

Howard Hughes Medical Institute and the Departments of Physiology and Biochemistry, University of California, San Francisco, California 94143, USA

Potassium channels comprise a diverse class of ion channels important for neuronal excitability and plasticity^{1,2}. The recent cloning of the *Shaker* locus from *Drosophila melanogaster* has provided a starting point for molecular studies of potassium channels³⁻⁵. Predicted *Shaker* proteins^{6,7} appear to be integral membrane proteins and have a sequence similar to the sequence of the S4 segment of the vertebrate sodium channel⁸, where the S4 segment has been proposed to be the voltage sensor⁸⁻¹¹. Expression studies in frog oocytes confirm that *Shaker* encodes a component of a potassium channel (the A channel) that conducts a fast transient potassium current¹². Here we report the isolation of complementary DNA clones from the mouse brain, the nucleotide sequences of which predict a protein remarkably similar to the *Shaker* protein. The strong conservation of the predicted protein sequence in flies and mammals suggests that these mouse clones encode a potassium channel component and that the conserved amino acids may be essential to some aspect of potassium channel function.

Sequences encoding the S4-like segment and the hydrophobic portion of a cDNA from the *Shaker* locus⁶, ShA1, were used to screen a mouse-brain cDNA library at reduced stringency (see Fig. 1). Four independent cDNA clones were sequenced. They appear to come from a single gene which we call MBK1 (MBK stands for mouse-brain K⁺ channel). All four cDNA clones contain the complete open reading frame shown in Fig. 1. Near the beginning of the single long open reading frame are two AUG potential start codons that are 6 bp apart. The sequences surrounding the first AUG agree most closely with the vertebrate consensus sequence for ribosome binding and translation initiation¹³. Therefore, we predict that the first AUG is the start of the MBK1 protein, which would result in a protein of 495 amino acids with a relative molecular mass (*M_r*) of 56,400 (56.4K). Because potential glycosylation sites are present, the actual *M_r* could be larger. Possible A-channel components of similar size (65K) have been identified in rat-brain synaptosomes by cross-linking studies using dendrotoxin¹⁵.

The predicted size and amino-acid sequence of the MBK1 protein are very similar to those of the A channel components

encoded by *Shaker* in *Drosophila*. Although multiple *Shaker* proteins are predicted⁷, the sequences showing amino-acid similarity with MBK1 are either in the common region or in regions that are highly conserved in all of the *Shaker* proteins. Overall, 65% of the amino acids are identical between the MBK1 protein and any of the predicted *Shaker* proteins. In contrast, the sodium channel from rat brain and the dihydropyridine (DHP) receptor¹⁶ from rabbit muscle are much larger, and show much less amino-acid similarity with MBK1 or the *Shaker* products. The strongest conservation among these four is in the S4-like sequence, which has been proposed to serve as a voltage sensor of the ion channel⁸⁻¹¹. The S4-like sequence contains basic residues (Arg or Lys) at every third position; in between are mainly hydrophobic residues. Whereas only one S4-like sequence is present in the MBK1 or *Shaker* product^{6,7}, there is one in each of the four internally homologous domains of the vertebrate sodium channel or DHP receptor^{8,16}. Moreover, no more than 10 or 11 residues are identical between the S4-like sequence of MBK1 and those of the vertebrate sodium channel or DHP receptor. In contrast, all 25 residues in the S4-like sequence are identical in MBK1 and the *Shaker* product (Fig. 2). Therefore, in terms of both size and sequence, the MBK1 protein resembles an A-channel component in *Drosophila* more strongly than the sodium channel or the DHP receptor in vertebrates.

How does the amount of conservation between MBK1 and *Shaker* compare with that observed in other ion-channel genes cloned from both flies and mammals? In a stretch of 63 amino acids, including the S4-like and predicted hydrophobic segment, H4, the sequences of MBK1 and *Shaker* are 97% identical (Fig. 2). At most 76% of the amino acids in analogous positions are identical between the sodium channels of *Drosophila* and rat¹⁷. Similarly, there is ~42% overall amino-acid identity between the nicotinic acetylcholine receptors in *Drosophila* and rat^{18,19}, compared with the 65% identity observed between *Shaker* and MBK1. Thus, among the ion channel genes that have been isolated from both *Drosophila* and mammals, the conservation between MBK1 and *Shaker* is the greatest. These considerations of size and sequence conservation strongly suggest that MBK1 encodes a component of a potassium channel, though expression or reconstitution in a heterologous system, such as the *Xenopus* oocyte, is required to determine the function of the MBK1 protein.

Three general observations can be made about the similarity between the predicted MBK1 protein and the *Shaker* product (Fig. 1). First, near either end of the protein there is little amino-acid similarity. Second, conservation is greater in the hydrophobic stretches (segments H1-6 and the S4-like sequence) than in the hydrophilic regions that separate them. Third, the highest conservation is observed in two stretches each of more than 50 amino acids (MBK1 residues 71-123 and 287-349) in which over 95% of the residues are identical between MBK1 and *Shaker*. One of these stretches corresponds to S4-like and H4 sequences, including the sequence in between; the other is in the hydrophilic sequence preceding the first hydrophobic sequence (H1).

In contrast to the strong homology at the level of the protein, the nucleotide sequences of MBK1 and *Shaker* are quite divergent, even in regions where the amino-acid sequence is highly conserved. For example, for the 25 residues in the S4-like sequence that are identical, the degeneracy of the genetic code would permit variation at 39 nucleotide positions. Thirty of these 39 nucleotides (77%) are in fact different between MBK1 and *Shaker* (Fig. 1). Because flies and mice diverged ~600 million years ago, this level of variation may be explained by the complete randomization of nucleotides at the degenerate positions^{20,21}. Thus the observed similarities between these two proteins probably reflect strong selective pressure to maintain amino acids essential for the protein's function.

One possible role for the conserved residues could be to

* Address from September 1988: Departments of Pharmacology and Medicine, University of Washington, and Seattle Veterans Administration Medical Center, 1660 S. Columbian Way, Seattle, Washington 98108, USA.

MBK1 1584 CTTTATTTTGCTGGCAATGCATTGTTGCATTGTGAATTTGGGGAGTGGCGAACCTGAAGCTTCAAGATCCATTAACCAACACAAAGCAAAAAA--3

Methods. Plaques ($\sim 10^6$) from a mouse-brain cDNA library (Clontech 1002) were screened using low-stringency hybridization²⁵. Two MBK1 cDNAs of 0.9 and 0.4 kb were isolated and used in turn to screen a mouse-brain cDNA library²⁶ at high stringency. Eleven MBK1 cDNA clones were isolated from $>10^6$ plaques. Four cDNAs of ~ 3 kb each were subcloned into M13 vectors. These, and recloned internal restriction fragments, were sequenced on both strands by the dideoxy-termination technique²⁷, using oligonucleotide primers and the T7 (Sequenase) or Klenow polymerases. The first 300 bp of 5' untranslated sequence was determined on only one strand. Amino-acid sequences were aligned using the Genalign program²⁸, except that the three carboxyl-terminal residues were recognized by eye. Nucleotide alignment was deduced from the amino-acid alignment. Sequence data in this publication have been submitted to EMBL/GenBank Libraries under the accession no. Y00305.

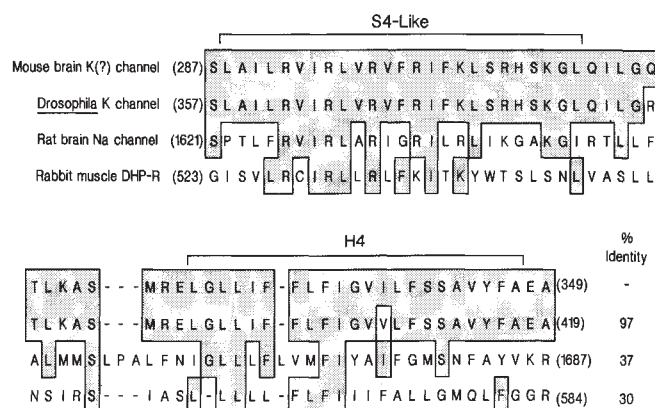


Fig. 2 Alignment of the proposed voltage sensor, the S4-like sequence, and the adjacent H4 sequence of the predicted MBK1 protein, the potassium channel from *Shaker*⁶, the rat brain Na channel II (ref. 8) and the rabbit DHP receptor¹⁶. Identical amino acids are boxed. The first and last amino acids of each sequence are numbered. The per cent identity relative to the MBK1 sequence is given for each protein on the lower right. Alignment was initially determined by the Genalign program²⁸, then refined by eye.

maintain a precise higher-order protein structure that has the characteristic ion selectivity and kinetic properties of the channel. For instance, the large number of perfectly conserved hydrophobic residues in the potential membrane-spanning regions suggests that some of these amino acids could contact or interact with other parts of the channel protein. In contrast to the near perfect conservation seen in the other predicted membrane spanning segments, one notices that conservative substitutions often occur at every third or fourth position in the H1-3 sequence. We therefore suggest that H1-3 form α -helices at the outer rim of the channel, exposing one face of the helix to a lipid environment where there might be less stringent selective pressures for absolute conservation.

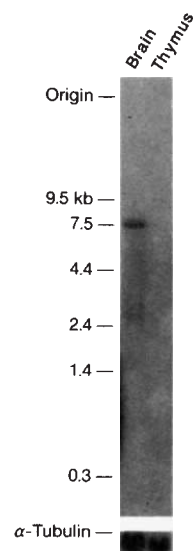
Another observation of possible structural significance is the conservation of a potential site for phosphorylation by cyclic AMP-dependent protein kinase (MBK1 residue 446, ref. 22). This conserved sequence is in a region near the carboxyl end that shows little overall amino-acid similarity, implying that the phosphorylation site may have some functional role subject to selective pressure. If this site is used *in vivo*, it is likely that the carboxyl terminus would be in the cytoplasm. The amino end is probably also cytoplasmic as no signal sequence is present at the amino terminus. Thus, although the hydropathy analysis of *Shaker* proteins indicates that there are six stretches of hydrophobic residues in addition to the S4-like sequence⁶, these polypeptides may be arranged in the membrane in such a way that both the amino and carboxyl termini are cytoplasmic.

The strong conservation may also allow one to identify amino acids that are important for channel functions. Possible candidates are residues in the hydrophilic sequence before H1, in the S4-like and hydrophobic sequences, in the sequence immediately following H6, at the phosphorylation site, as well as the last three residues at the carboxyl terminus. The function of these conserved amino acids may be studied by mutagenesis.

A single band of ~8 kilobases (kb) hybridized with the MBK1 sequence (Fig. 3) on a Northern blot of mouse-brain poly-A⁺ RNA. One of the MBK1 cDNAs had a poly(A) tail at its 3' end and, 24 bp 5' from this, a hexanucleotide sequence (ATTAAA) thought to signal polyadenylation²³. If this polyadenylation site is used *in vivo*, we predict that the 5' untranslated portion of the MBK1 transcript is >6 kb. Similarly large transcripts with small coding regions have been seen in other channel genes^{7,19,24}. The function, if any, of these large and presumably untranslated regions is unknown. Although we cannot exclude the possibility

Fig. 3 Northern blots of poly(A)⁺ RNA from mouse brain and thymus probed with an MBK1 cDNA. The size of RNA molecular weight standards (BRL) are indicated in kb at the left. The blots were reprobed with chicken α -tubulin cDNA (ref. 29) to control for degradation.

Methods. RNA was isolated from total mouse brain and thymus using guanidinium isothiocyanate and the poly(A)⁺ fraction was a gift from D. Littman²⁶. An estimated 5 μ g brain and thymus poly(A)⁺ RNA were run on formaldehyde gels, blotted to Gene-screen nylon membranes and probed with the MBK1 sequence that was labelled using random primers (specific activity >10⁹ d.p.m. per μ g) as described⁷.



that multiple MBK1 transcripts occur in the mouse brain but are not detected as distinct bands on our blots, the appearance of a single transcript from MBK1 differs strongly from the observed transcription of *Shaker*, which produces a large family of poly(A)⁺ RNAs in *Drosophila*⁷. It remains to be determined whether MBK1 encodes a single gene product that alone fulfils its functional role in brain tissue or whether MBK1 belongs to a family of closely related potassium channel genes in the mouse.

A possible potassium channel component from the mouse brain has been cloned based on its strong structural homology with the A-channel component from the *Shaker* locus. This represents the first case in which the characterization of an ion channel in *Drosophila* has allowed a similar channel to be isolated from mammals. The comparison of amino acids conserved in MBK1 and *Shaker* may be useful for designing and constructing chimeric or *in vitro* mutagenized channels that could help to define structures important for channel function.

We thank D. Littman for the mouse brain cDNA library and poly(A)⁺ RNA, D. Lantz and M. Doherty for technical help and L. Schulte for preparing the manuscript. This study was supported by the Howard Hughes Medical Institute and NIH. Y.N.J. and L.Y.J. are Hughes Investigators.

Received 16 February; accepted 14 March 1988.

- Hille, B. *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, Massachusetts, 1984).
- Byrne, J. H. *Physiol. Rev.* **67**, 329-439 (1987).
- Papazian, D. M., Schwarz, T. L., Tempel, B. L., Jan, Y. N. & Jan, L. Y. *Science* **237**, 749-753 (1987).
- Kamb, A., Iverson, L. E. & Tanouye, M. A. *Cell* **50**, 405-413 (1987).
- Baumann, A. et al. *EMBO J.* **6**, 3419-3429 (1987).
- Tempel, B. L., Papazian, D. M., Schwarz, T. L., Jan, Y. N. & Jan, L. Y. *Science* **237**, 770-775 (1987).
- Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, Y. N. & Jan, L. Y. *Nature* **331**, 137-142 (1988).
- Noda, M. et al. *Nature* **320**, 188-192 (1986).
- Greenblatt, R. E., Blatt, Y. & Montal, M. *FEBS Lett.* **193**, 125-134 (1985).
- Guy, H. R. & Seetharamulu, P. *Proc. natn. Acad. Sci. U.S.A.* **83**, 508-512 (1986).
- Catterall, W. A. *Rev. Biochem.* **55**, 953-985 (1986).
- Timpe, L. C. et al. *Nature* **331**, 143-145 (1988).
- Kozak, M. *Cell* **44**, 283-292 (1986).
- Hubbard, S. C. & Ivatt, R. J. A. *Rev. Biochem.* **50**, 555-583 (1983).
- Mehrabani, F., Breeze, A. L. & Dolly, J. O. *FEBS Lett.* **174**, 116-122 (1984).
- Tanabe, T. et al. *Nature* **328**, 313-318 (1987).
- Salkoff, L. et al. *Science* **237**, 744-749 (1987).
- Hermans-Borgmeyer, I. et al. *EMBO J.* **5**, 1503-1508 (1986).
- Bossy, B., Ballivet, M. & Spierre, P. *EMBO J.* **7**, 611-618 (1988).
- Perder, F. et al. *Cell* **20**, 555-566 (1980).
- Kimura, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 454-458 (1981).
- Kemp, B. E. et al. *J. biol. Chem.* **252**, 4888-4894 (1977).
- Goeddel, D. V. et al. *Nature* **290**, 20-26 (1981).
- Grennington, G. et al. *Nature* **328**, 215-220 (1987).
- Nathans, J., Thomas, D. & Logness, D. S. *Science* **232**, 193-202 (1986).
- Lomberg, N., Gettner, S. N., Lacy, E. & Littman, D. R. *Molec. cell. Biol.* (in the press).
- Sanger, F. et al. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Sobel, E. & Martinez, H. M. *Nucleic Acids Res.* **14**, 363-374 (1985).
- Cleveland, D. W. et al. *Cell* **20**, 95-105 (1980).

T cells can distinguish between allogeneic major histocompatibility complex products on different cell types

Philippa Marrack* & John Kappler†

Howard Hughes Medical Institute at Denver, Department of Medicine, National Jewish Center for Immunology & Respiratory Medicine, and Departments of Biochemistry, Biophysics, Genetics*, Microbiology and Immunology† and Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80206, USA

In the response of T cells to foreign antigens, the ligand for the T cell α/β receptor is presented on a cell surface as a fragment of antigen complexed to one of the membrane molecules encoded in the major histocompatibility complex (MHC)¹⁻⁵. The receptor apparently interacts via its variable elements (V β , D β , J β , V α and J α) with residues within both the antigen and MHC portion of the ligand. The frequency of T cells responding to a conventional antigen plus self MHC is usually quite low, presumably reflecting the relative rarity of receptors with the particular combination of variable elements to match the antigen/MHC ligand. T cells also respond to allogeneic forms of MHC molecules in the absence of added antigen⁶. In this case the frequency of responding T cells is very high. One hypothesis to explain this observation is that, in the absence of foreign antigen, MHC molecules are complexed to a large array of peptides derived from self-proteins⁷. In this case the combination of the polymorphic MHC amino acid residues and many different self peptides presents so many possible ligands that the likelihood of recognition by a given T cell receptor is quite high. The recent crystallography experiments which revealed a dramatic binding cleft on the face of a human MHC molecule⁵ have given impetus to this view, but as yet there is no direct supporting evidence. We have recently described a close association between murine T cell receptors utilizing the V β 17a element and reactivity to various allogeneic forms of the murine MHC molecule, I-E (ref. 8). In this paper, we show that this I-E ligand is detected on B cells, but not on I-E⁺ macrophages or fibroblasts expressing a transfected I-E gene. These results strongly suggest a B cell specific product combines with I-E to form the allogeneic ligand for V β 17a⁺ receptors and thus support the concept of alloreactivity described above.

We compared the response of V β 17a⁺ T cell hybridomas against I-E on transfected fibroblasts, unseparated spleen cells and B cell lymphomas. The averaged results of these experiments are shown in Table 1.

The three V β 17a⁺ T cell hybridomas responded to I-E^k on C3H spleen cells and the B cell lymphoma CH12. They did not respond to I-E^k on the transfected fibroblasts DCEK or CA36.1.3. Similar results have been obtained with more than ten other V β 17a⁺ T cell hybridomas tested and several other fibroblast lines expressing I-E^b or I-E^d. By contrast, a control hybridoma 2B4.6, specific for pigeon cytochrome *c* peptide 88-104 bound to I-E^k, responded to this combination on all I-E^k-bearing cells tested. The failure of V β 17a⁺ T cells to respond to I-E^k on transfected fibroblasts was not caused by low I-E^k expression on these cells since, as shown in Table 1, DCEK and CA36.1.3 actually bear considerably more I-E^k per cell than C3H spleen cells. Fibroblast cells are larger than splenic B cells, but they are about the same size as CH12 cells, so this is not a matter of I-E density.

We also screened the V β 17a hybridomas for their response to other cell lines bearing various alleles of I-E. We found that the T cells responded to I-E on any B cell lymphoma or hybridoma we tested, but that they failed to react to I-E^d on a macrophage tumour line, P388D1. This macrophage line expresses very low levels of Class II molecules constitutively, but high levels can be induced by 48 h culture in interferon γ

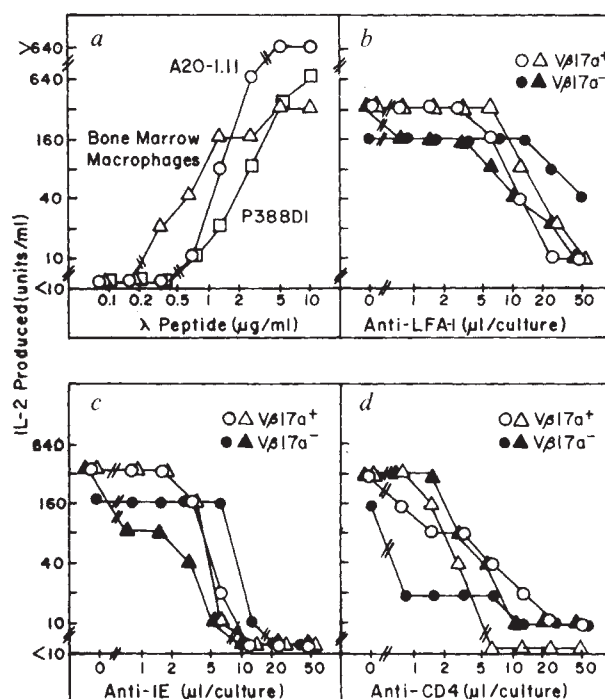


Fig. 1 Sensitivity of responses of V β 17⁺ and V β 17a⁻ T cell hybridomas to peptide concentration and antibody inhibition. *a*, The T cell hybridoma, LFE1 was cultured for 24 h with varying concentrations of λ repressor peptide (12-26) and the I-E^d presenting cells: A20-1.11 B cell lymphoma (○), IFN γ -induced P388D₁ macrophage cell line (□) and IFN γ -induced macrophages grown from BALB/c bone marrow (Δ). The magnitude of response was measured by assaying the IL-2 secreted¹⁹. *b-d*, T cell hybridomas and A20-1.11 antigen presenting cells were cultured, with 10 μ g ml⁻¹ of λ repressor 12-26 where required, for 24 h, in the presence of various dilutions of culture supernatants of the following monoclonal antibodies: *b*, anti-LFA-1 (121/7, the gift of Dr I. Trowbridge²³); *c*, anti-I-E^d (M5/114 gift of T. Springer²⁴); *d*, anti-CD4 (GK1.5, the gift of Dr F. Fitch²⁵). Responses of the following T cell hybridomas were measured by assaying the IL-2 secreted¹⁹: Q023-26.6 (V β 17a⁺, I-E-reactive) (○); KQ23-27.7 (V β 17⁺, I-E-reactive) (Δ); LFE1 (V β 17a⁻, λ rep./I-E-reactive) (▲); LFSE1 (V β 17a⁻, λ rep./I-E-reactive) (●).

(IFN γ)⁹. Our V β 17a⁺ cell lines failed to respond to P388D1 with or without prior IFN γ induction in spite of the fact that they responded well to I-E^d on the B cell lymphoma A20-1.11 or on BALB/c spleen cells (Table 2). These T cell hybridomas also failed to respond to I-E^d on another macrophage cell line WeHi 3, with or without IFN γ induction (data not shown). A control V β 17a⁻ hybridoma specific for I-E^d, CG4 (ref. 10), and several T cell hybridomas specific for antigenic peptides plus I-E^d (for example LFE1) responded, in some cases very well, to induced P388D1 and the other cell types tested.

These results might have several explanations. The first, that the V β 17a⁺ hybridomas require high levels of surface I-E for activation, whereas other T cells are less I-E density dependent, would seem to be eliminated by our use of transfected fibroblasts and IFN γ -induced P388D1 as test cell lines, as both these cell types bear high levels of surface I-E (certainly greater than those on some of the cell types to which the V β 17a⁺ cells do respond). A second and related possibility is that V β 17⁺ receptors have low affinity for I-E, whereas the receptors on all the control cell lines we tested are of high affinity. If this were the case, the response of V β 17a⁺ cells would be particularly dependent on synergy in target cell binding between T cell receptors and 'accessory molecules' such as CD4 or LFA-1. This idea is of particular concern in the interactions of the T cell hybridomas with I-E on mouse fibroblasts, since such interactions are known

Table 1 I-E^k transfected into a fibroblast cell line is not recognized by Vβ17a⁺ T cell hybridomas

T cell hybridoma	Vβ expressed	Ag added	IL-2 produced in response to I-E ^k antigen presenting cells (units ml ⁻¹)			
			DCEK transfected fibroblast [148]*	CA36.1.3 transfected fibroblast [151]*	CH12 B cell lymphoma [48]*	C3H/HeJ spleen cells, [10]*
2Q23-34.7.9	17a	None	—	—	1940 (1,150–3,260)	430 (230–830)
Q023-26.6	17a	None	—	—	17 (10–30)	840 (560–1,280)
2S23-37.9	17a	None	—	—	320 (190–550)	46 (36–59)
2B4.6	3	pigeon cyt. c 88–104	1,690 (1,190–2,400)	190 (94–410)	280 (120–640)	240 (170–350)

The cell line DCEK Hi7 (DCEK), produced by transfection of thymidine kinase⁻ L cells (from C3H mice) with cDNA clones for the α and β chains of IE^k (ref. 17), was the gift of R. Germain. CA36.1.3, produced by transfection of similar L cells with genomic clones for E_α^d and E_β^k chains, was the gift of B. Malissen. CH12 is a B cell lymphoma derived from the B10.A.H-4^b IE^k-expressing strain¹⁸ and was the gift of G. Haughton. Three different Vβ17a⁺ T cell hybridomas were used: 2Q23-34.7.9, produced from anti-Vβ17a-stimulated SWR T cell blasts⁷; Q023-26.6, produced from chicken ovalbumen-primed SWR T cell blasts¹⁹ and 2S23-37.9, produced from anti-Vβ17a-stimulated SJL T cell blasts⁸. The three Vβ17a⁺ T cell hybridomas, reactive with various I-E alleles including k and d, and 2B4.6 (ref. 20), specific for pigeon cytochrome c 88–104/IE^k (the gift of S. Hedrick) were added at 10⁵ cells per assay well to 10⁵ fibroblast or CH12 cells, or 10⁶ spleen cells from C3H/HeJ mice. Pigeon cytochrome c peptide 88–104 was added at 10 μg ml⁻¹ to 2B4.6 stimulation cultures. After 24 h supernatants were harvested and titrated to measure interleukin-2 (IL-2) quantities produced¹⁹. Results shown are the geometric means and standard error ranges for four or five assays.

* Relative amount of I-E^k per cell determined by cytofluorographic analysis after reaction with biotinylated anti-I-E^k (Y17, the gift of Dr C. A. Janeway) and phycoerythrin-conjugated avidin. —, <10 units per ml IL-2 (the minimum detectable in this assay).

Table 2 A macrophage cell line does not present I-E^d to Vβ17a⁺ T cell hybridomas

T cell hybridoma	Vβ expressed	Ag added	IL-2 produced in response to I-E ^d antigen presenting cells (units ml ⁻¹)		
			P388D1 macrophage cell line	A20 B cell lymphoma	BALB/a spleen cells
2Q23-34.7.9	17a	None	—*	180 (125–260)	2,560 (1,540–4,290)
Q023-26.6	17a	None	—	502 (399–631)	832 (443–1,560)
2S23-37.9	17a	None	—	44 (29–65)	53 (30–93)
KQ23-27.7	17a	None	—	400 (348–465)	1,610 (1,280–2,020)
CG4	Not 17a	None	80 (58–111)	40 (28–56)	290 (174–485)
LFE1	Not 17a	λ rep 12–26	320 (160–640)	905 (495–1,650)	2,040 (1,830–3,240)

Class II expression on P388D1 cells was induced by incubation of 5 × 10⁴ P388D1 cells in 0.1 ml medium in microtitre wells with 1,000 units per ml IFNγ (ref. 9). After 2 days these wells were washed free of IFNγ, and 10⁵ of the relevant hybridoma T cells added. T cell hybridomas were also challenged with 10⁵ A20-1.11 (ref. 21) or 10⁶ BALB/c spleen cells. Four Vβ17a hybridomas with specificities for various alleles of I-E including d were used. Three of these are described in the footnote to Table 1; KQ23-27.7 was produced from anti-Vβ17a stimulated (AKR × SWR)F1 T cell blasts. CG4 (ref. 10), specific for I-E^d, was the gift of Dr H. Grey, and LFE1, specific for λ repressor peptide 12–26/IE^d, was the gift of Dr M. Gelfer (λ repressor peptide 12–26 was added at 10 μg ml⁻¹ to LFE1 stimulation cultures). IL-2 production was measured as described in the legend to Table 1. Results shown are the geometric means and standard error ranges of between 3 and 20 assays. —*, ≤2.5 units per ml IL-2 (minimum detectable in these assays).

to be LFA-1 independent¹¹. T cell interactions with P388D1 utilize LFA-1 (personal observation), however, and therefore such an explanation cannot account for the failure of Vβ17a hybridomas to recognize this macrophage cell line.

Nevertheless, two types of experiment were done to check this latter possibility. In the first, T cell hybrids specific for antigenic peptides (cytochrome c or λ repressor) plus I-E were tested for their ability to respond to different concentrations of peptide presented by spleen cells, B lymphoma cells, bone-marrow-derived macrophages and transfected fibroblasts or P388D1. As illustrated in Fig. 1a there was no appreciable difference between the antigen-presenting activities of bone-marrow-derived macrophages, P388D1 or A20-1.11 to the T cell hybridoma LFE1. Similar results were obtained using I-E^k-presenting lines and 2B4.6, a T cell hybridoma specific for a pigeon cytochrome c peptide. In a second type of experiment, T cell hybrids were tested for sensitivity to inhibition of their responses by anti-LFA-1, anti-CD4 and anti-IE. Such inhibitions are usually taken, in lieu of any other currently available method, to be relative measures of the affinity of the receptors on responding T cells for their targets. As shown in Fig. 1b–d, the responses to I-E of the Vβ17a⁺ cells were not significantly more sensitive to inhibition by any antibody tested than those of control Vβ17a⁻ hybridomas. Thus the cell type-specific differences in responsiveness of Vβ17a⁺ and Vβ17a⁻ T cells cannot be explained by

differential dependence on a known accessory molecule.

The finding that Vβ17a⁺ T cell hybridomas fail to respond to I-E on macrophage tissue culture lines prompted us to compare their response to macrophages isolated directly from mice and macrophages grown over the course of two weeks from bone marrow cells in the presence of colony stimulating factors and, for the last three days, IFNγ. One representative experiment out of the three performed is shown in Table 3. The Vβ17a⁺ cell lines responded well to I-E^d or I-E^k on spleen cells and peritoneal macrophages. They failed, however, to respond to I-E^d or I-E^k on macrophages derived by extended culture from bone marrow precursors. Control Vβ17a⁻ hybridomas responded to I-E^d or I-E^k, in the presence of antigen where necessary, regardless of the type of the antigen presenting cells. In addition, bone-marrow-derived macrophages presented exogenously added peptides to I-E-restricted hybridomas as effectively as spleen cells (Fig. 1a).

Taken together, these results suggest that Vβ17a⁺ receptors can distinguish I-E on B cells and peritoneal macrophages from the same I-E allele on fibroblasts and cultured macrophage lines. This phenomenon does not seem to be related to the density of I-E on the different cell types, nor to the normal or transformed phenotype of the presenting cells nor to the role of some known accessory molecule. The real explanation for our results may derive from hypothesis put forward some years ago by Matzinger

Table 3 V β 17a⁺ T cell hybridomas do not respond to I-E on bone marrow-derived macrophages

T cell hybridoma	Spleen cells	IL-2 (units ml ⁻¹) produced in response to:				
		C3H/HeJ (I-E ^k) Peritoneal macrophages	Bone marrow macrophages	Spleen cells	BALB/c (I-E ^d) Peritoneal macrophages	Bone marrow macrophages
2Q23-34.7.9	2,560	2,560	—*	1,280	320	—
Q023-26.6	5,120	2,560	—	1,280	1,280	—
2S23-37.9	40	20	—	40	80	—
2B4.6	10,240	2,560	1,280	ND	ND	ND
CG4	ND	ND	ND	320	20	10
LFE1	ND	ND	ND	1,280	160	80

Spleen cells, peritoneal macrophages and bone marrow cells were harvested from normal C3H/HeJ or BALB/c mice. Macrophages were induced from bone marrow cells by 11 days' culture in macrophage colony stimulating factor as described²². Class II antigens were subsequently induced on these cells by 3 days' culture in IFN γ . Peritoneal macrophages were prepared from peritoneal washings by isolation of plastic adherent cells. T cell hybridomas were challenged with IE-bearing cells, in the presence of exogenously added antigenic peptides where necessary, and their responses measured as described in the legends to Table 1 and Table 2. The results shown are those from one of three similar assays.

—*, <10 units per ml IL-2 (minimum detectable in this assay). ND, not determined.

and Bevan⁷. They suggested that recognition of allogeneic MHC by a T cell may in fact represent recognition of a foreign MHC molecule associated with any one of a multitude of self proteins which might not even vary within the species. According to this hypothesis, T cells specific for allogeneic MHC are very frequent because so many combinations of self protein and MHC may exist as potential ligands for T cell receptors. Recent work on recognition of antigen by T cells, and on the structure of MHC supports a variant of this idea²⁻⁵. It is now known that in normal immune responses peptides, derived from foreign antigens, bind to self MHC proteins²⁻⁴, and it is this complex which is recognized by T cells. Moreover it has also been shown that peptides derived from the host itself can bind to self MHC, compete for binding with peptides derived from antigen and, under appropriate circumstances, be recognized by T cells¹². For example it has been shown that peptides derived from one of the alleles of mouse globin can be recognized in association with MHC by T cells derived from mice expressing another mouse globin allele (P. Allen, personal communication). It is therefore likely that recognition by T cells of allogeneic MHC also involves recognition of peptides bound to that MHC. These peptides may not vary within the species, but may vary from cell type to cell type, because of tissue-specific expression of or catabolism of the proteins from which they are derived.

With these findings in mind, we would like to suggest three different explanations for the observations reported in this paper. The first hypothesis, and the one we currently prefer, is based on the idea that I-E-reactive, V β 17a⁺ T cell hybridomas are in fact specific for I-E complexed with a particular peptide or group of peptides. This peptide(s) must be specific to mouse B cells, and must not be expressed by mouse macrophage or fibroblast lines, either because the protein(s) from which it is derived is B cell-specific, or because of differences in proteolytic breakdown pathways between different cell types. This determinant may be present on peritoneal macrophages although absent from bone-marrow-culture-derived macrophages, because of *in vivo* transfer of peptide or protein from other cells to macrophages, a phenomenon already documented for the globin alleles described above (P. Allen, personal communication).

This hypothesis predicts that V β 17a-bearing receptors must recognize the same peptide or group of peptides bound by different I-E alleles. This is possible, as although some peptides can be bound by only one or a few of all alleles of a particular MHC molecule there is evidence that some peptides can bind more promiscuously, to virtually all alleles of the same type of MHC molecule^{3,13}.

A second explanation for the observations in this paper could be the involvement of some hitherto unknown B cell specific accessory molecule in V β 17a⁺ T cell responses. This molecule

might act to increase the strength with which otherwise non-reactive, low affinity V β 17a⁺ T cells interact with I-E⁺ targets. This explanation does not fit as well as the first with the data presented in this paper for two reasons. First, V β 17a⁺ T cell receptors appear to have about the same avidity for I-E, at least as measured by antibody blocking, as V β 17a⁻ receptors which do not require this hypothetical B cell-specific accessory molecule for function. Secondly macrophages assayed immediately after isolation from animals can present IE to V β 17a⁺ T cells, whereas cultured macrophages cannot. If this second hypothesis were correct we would have to invoke an additional premise, that macrophages *in vivo* but not *in vitro* can synthesize the needed accessory molecule.

Finally, it is possible that I-E polypeptides expressed on B cell surfaces are not all exactly the same as those on macrophages and fibroblasts. Perhaps a small amount of a B cell I-E is differently glycosylated, or because of different RNA splicing is of different amino acid sequence, or is bound by B cell-specific proteins which alter its conformation. Again a hypothesis of this type requires that *in vivo* and *in vitro* macrophages synthesize different proteins. At present neither of these two last possibilities can be completely dismissed. Our current experiments are aimed at identifying the correct explanation.

Several other points are worth noting. First, these results support the idea that MHC molecules on different cell types may not be recognized in the same way by T cells, a finding that has been made before, perhaps for similar reasons^{14,15}. This conclusion may have important consequences in the understanding of autoimmunity and mechanisms of graft rejection. Secondly we have recently found another V β product which, like V β 17a, confers a particular reactivity upon T cell receptors of which it is a component¹⁶. This V β , (8.1) imparts reactivity for MIs/Class II. MIs^a is expressed in B cells and not in macrophages, and thus in many ways the pattern of tissue reactivity of V β 17a⁺ T cells for IE and V β 8.1⁺ T cells for MIs/Class II are similar, a finding which must have implications for the understanding of the T cell repertoire.

We thank Drs M. Geffer, R. Germain, H. Grey, S. Hedrick and B. Malissen for the gifts of cell lines used in these studies, Drs F. Fitch, C. Janeway, T. Springer and I. Trowbridge for monoclonal antibody producing cell lines and Drs D. Riches and D. Laszlo for help with the production of bone marrow-derived macrophages. We also thank Ella Kushnir for technical assistance and Donna J. Thompson for secretarial help. This work was supported by research grants from the USPHS and American Cancer Society.

Received 10 February; accepted 15 March 1988.

1. Zinkernagel, R. & Doherty, P. J. *exp. Med.* **141**, 1427-1436 (1975).
2. Babbitt, B., Allen, P., Matsueda, G., Haber, E. & Unanue, E. *Nature* **317**, 359-361 (1985).

3. Buus, S., Sette, A., Colon, S., Miles, C. & Grey, H. *Science* **235**, 1353-1358 (1987).
4. Townsend, A. R. M. *et al.* *Cell* **44**, 959-968 (1986).
5. Bjorkman, P. *et al.* *Nature* **329**, 512-518 (1987).
6. Fischer-Lindahl, K. & Wilson, D. *J. exp. Med.* **145**, 508-522 (1977).
7. Matzinger, P. & Bevan, M. J. *Cell. Immun.* **29**, 1-5 (1977).
8. Kappler, J. *et al.* *Cell* **49**, 263-271 (1987).
9. Vitetta, E. *et al.* *J. exp. Med.* **162**, 1726-1731 (1985).
10. Coeshott, C. M. *et al.* *J. Immun.* **136**, 2832-2838 (1986).
11. Golde, W. *et al.* *J. exp. Med.* **161**, 635-640 (1985).
12. Babbitt, B. P., Matsueda, G. R., Haber, E., Unanue, E. R. & Allen, P. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4509-4513 (1986).
13. Lynch, D. H., Cole, B. C., Bluestone, J. A. & Hodes, R. J. *Eur. J. Immun.* **16**, 747-751 (1986).
14. Manca, F. *et al.* *Transplantation* **36**, 670-674 (1983).
15. Minami, M., Kawasaki, H., Taira, S. & Nariuchi, H. *J. Immun.* **135**, 111-116 (1985).
16. Kappler, J., Staerz, U., White, J. & Marrack, P. *Nature* **332**, 35-40 (1988).
17. Ronchese, F., Schwartz, R. H. & Germain, R. N. *Nature* **329**, 254-256 (1987).
18. Arnold, L. W. *et al.* *J. Immun.* **131**, 2064-2068 (1983).
19. Kappler, J., Skidmore, B., White, J. & Marrack, P. *J. exp. Med.* **153**, 1198-1214 (1981).
20. Hedrok, S., Cohen, D., Nielsen, E. & Davis, M. *Nature* **301**, 149-153 (1984).
21. Kim, K., Kanellopoulos-Langevin, C., Merwin, R., Sachs, D. & Asofsky, R. *J. Immun.* **122**, 549-554 (1979).
22. Stiert, C. C. in *Handbook of Experimental Immunology* Vol. 2 (eds Weir, D. M., Herzenberg, L. A., Blackwell, C. & Herzenberg, L. A. 44.1-44.17 (Blackwell, Oxford 2, 1987).
23. Trowbridge, I. & Omary, M. *J. exp. Med.* **154**, 1517-1524 (1981).
24. Bhattacharya, A., Dorf, M. E. & Springer, T. A. *J. Immun.* **127**, 2488 (1981).
25. Dialynas, D. *et al.* *J. Immun.* **131**, 2445-2451 (1983).

Suppression of experimentally induced autoimmune encephalomyelitis by cytolytic T-T cell interactions

Deming Sun*, Yufen Qin*, Johanna Chluba†, Jörg T. Epplen†, & Hartmut Wekerle*†

* Max-Planck Society, Clinical Research Unit for Multiple Sclerosis, D-8700 Würzburg, FRG

† Max-Planck-Institute for Psychiatry, D-8033 Martinsried/Munich, FRG

Down-regulatory phenomena have been described in several experimental models of tissue-specific, T-cell-mediated autoimmunity. For example, resistance to active induction of experimental autoimmune encephalomyelitis (EAE) can be induced by pretreating animals with non-pathogenic inocula of autoantigen¹ or effector cells^{2,3}. Moreover, animals that have recovered from one EAE episode are resistant to subsequent induction of EAE⁴⁻⁶. In some models, resistance to EAE has been transferred with immune cells to naive recipients⁷⁻¹². These experiments, which were based on transfers of unseparated immune cell populations, are difficult to interpret. Immune suppression circuits are known to be complex and involve various distinct cellular subsets¹³. To further complicate the issue, resistance to EAE can be transferred not only by suppressor cells, but also by encephalitogenic effector cells injected in 'subclinical' doses^{2,12}. We describe now the isolation of homogenous T lymphocyte lines from the spleens of Lewis rats that had recovered from T-cell-mediated EAE (tEAE) caused by the MBP-specific T cell line S1. These spleen-derived T line cells express the CD8 phenotype and specifically respond to determinants on the inducing S1 line, but not to the autoantigen MBP. Furthermore, the anti-S1 cells selectively lyse the encephalitogenic S1 T line *in vitro* and efficiently neutralize their encephalitogenic capacity *in vivo*.

EAE transferred by activated encephalitogenic T cell lines (tEAE) has a typical monophasic course. After a symptom-free interval of about 3 days post-inoculation (p.i.) ascending paralysis develops during the subsequent few days and may lead to death. Surviving animals, however, recover from clinical tEAE after disease durations roughly proportional to the dose of pathogenic cells injected. Spontaneous relapses have not been observed in rat tEAE. We have induced sublethal EAE with a MBP-specific T cell line (S1) selected from a Lewis rat preimmunized with MBP in Freund's complete adjuvant. Like all our antigen specific T lymphocyte lines, line S1 recognizes the encephalitogenic epitope on MBP (sequence 68-88) in the con-

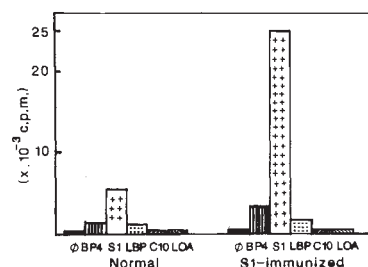


Fig. 1 Response pattern of post-tEAE Lewis spleen T cells. The response pattern was assessed in 96-well flat-bottomed microtitre plates by confronting 2×10^5 nylon-wool enriched post-tEAE T cells with 4×10^4 irradiated syngeneic CD4⁺ T cells (from the clones or lines indicated) in the absence of the relevant antigens. After 72 h the cultures were pulsed with $0.5 \mu\text{Ci } ^3\text{H}$ -thymidine for 18 h, harvested and processed for determination of isotope uptake. The reactivity of T cells from a normal Lewis spleen was compared in parallel. T cell lines/clones BP4, S1, LBP and C10 are specific for Guinea pig MBP sequence 68-88 (in the context of RT1.B (I-A) gene products); they all are encephalitogenic. L.O.A recognizes chicken ovalbumin, also in the context of RT1.B products.

text of Ia determinants of the MHC and expresses the CD4 phenotype.

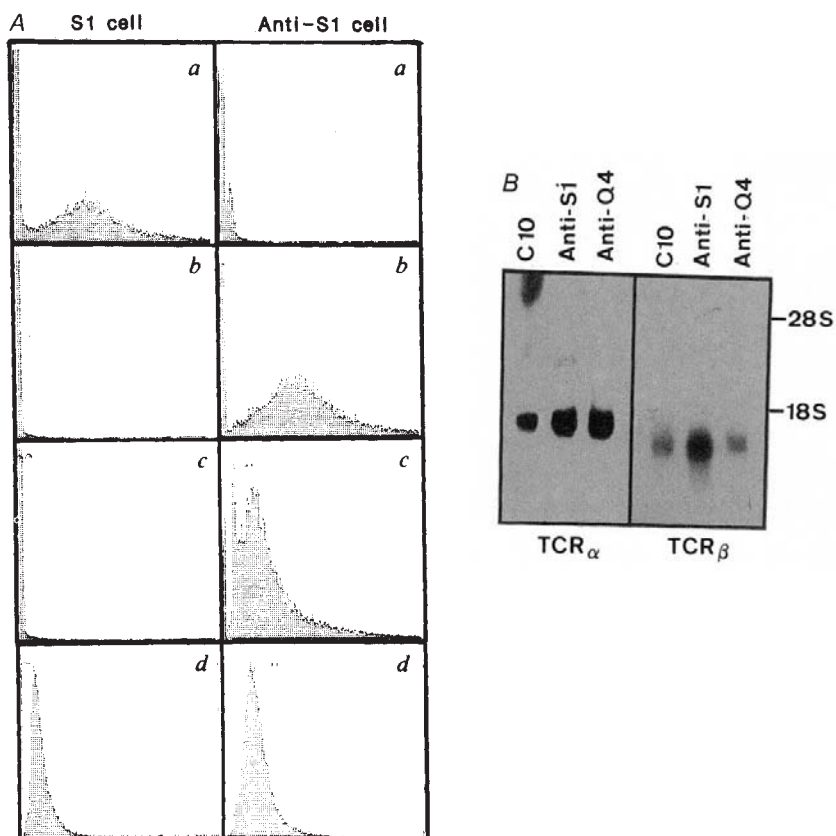
To investigate whether S1-specific counter-regulatory immune responses by the host immune system have a role in spontaneous remission after tEAE, we isolated T lymphocytes from spleens of Lewis rats that had recovered from S1-mediated tEAE. These T cell populations were confronted *in vitro* with irradiated cells of line S1 or with other syngeneic T lines recognizing either MBP or control antigens. Figure 1 shows that splenic T cells from animals that had recovered from S1-induced EAE proliferated strongly and specifically upon contact with S1 target cells. The proliferative response against unrelated T lines was low, as was the response of naive, normal splenic T cells against any of the target T line cells. This anti-S1 response was seen in the absence of MBP. Addition of the autoantigen did not markedly affect the reaction (data not shown).

Activated lymphoblasts were separated from the mixed cultures by density gradient centrifugation, and expanded in the presence of interleukin 2 (IL-2). Cytofluographic analyses (Fig. 2A) showed that practically all cells of anti-S1 lines expressed the CD8 phenotype. They were bound by monoclonal antibodies (mAbs) OX8 and OX19, but not by W3/25. In contrast to encephalitogenic CD4⁺ T line cells, the CD8⁺ anti-S1 cells expressed high levels of Ia antigens on their surface. Full-length message for the T cell receptor α - and β -chain was demonstrated (Fig. 2B) for the CD8⁺ anti-S1 line and for an analogous but independent CD8⁺ Lewis T line (anti-Q4) which is specific for the encephalitogenic Lewis T line Q4 (reactive against Guinea pig MBP sequence 68-88 in context of RT1.B¹ products). Full-length messenger RNA was also detected, as expected, in the encephalitogenic CD4⁺ Lewis T clone C10 (see legend to Fig. 1).

Anti-S1 T cell lines were monospecific for determinants on S1-line cells. Like the original post-tEAE splenic T populations, selected anti-S1 line cells proliferated in the presence of S1, but not of control syngeneic T line cells. They were, moreover, completely non-responsive to MBP presented by classical antigen presenting cells. Activation of anti-S1 T line cells was not significantly blocked by mAbs against class I (OX18) or class II MHC antigens (OX3, OX6 and OX17), nor by mAbs binding to the differentiation marker, OX8 (data not shown). This is in contrast to MBP-specific T cells, whose activation is effectively blocked by antibodies to Ia, as well as by W3/25 mAbs¹⁴. The CD8⁺ T cell subset is reputed to include suppressor as well as cytotoxic T lymphocyte populations¹⁵ and functional assays revealed that both functions are exerted by anti-S1 T lines. Isotope release-assays showed that anti-S1 cells efficiently and

Fig. 2 A, Membrane phenotype of anti-S1 and S1 T lines. T lymphocyte line S1 was selected from the spleen of a MBP-immune Lewis rat according to a standard protocol⁸. S1 cells express the CD4 phenotype (left side) and recognize MBP in the context of Ia determinants. Freshly activated cells of either S1 or anti-S1 lines were stained with monoclonal antibodies W3/25 (a), OX8 (b), Ia-specific OX6 (c) and OX19 (d), plus FITS-conjugated goat anti-mouse Ig antibody. Fluorescence intensity was determined on a Cytofluorograph (Ortho). B, Expression of T cell receptor α - and β -chain mRNA in the CD8⁺ T cell lines anti-Q4 and anti-S1. Line anti-Q4 was selected from the spleen of a Lewis rat which had been recovered from EAE mediated by T line Q4 (specific for GP MBP sequence 68-88 and restricted by RT1B¹ products). As a positive control, RNA of the T cell clone C10 (see legend to Fig. 1) was included. The positions of ribosomal RNAs are indicated. In all RNAs full-length T cell receptor α - and β -chain message (1.5 and 1.3 kilobase) is expressed.

Methods. Anti-S1 T lines were selected from the spleen of 12-week-old Lewis rats, which had been injected i.v. 3 weeks before with 2 millions of freshly activated S1 cells and had recovered from severe tEAE. Splenic T cells were enriched by filtration through a nylon wool column, and subsequent absorption for 18 h on plastic dishes. Post-tEAE splenic T cells (2×10^7) were co-incubated with 4×10^6 irradiated S1 cells in 10 ml RPMI-1640 medium containing 10% FCS. After 3 days, activated proliferating lymphoblasts were isolated by Ficoll Hypaque density gradient centrifugation and cultured in IL-2 containing medium. Total cellular RNA from the T cells was prepared by guanidinium thiocyanate extraction and caesium chloride cushion centrifugation as described previously²⁴. Gel electrophoresis and in-gel hybridization was carried out essentially according to Hochgeschwender *et al.*²⁵ using the ³²P-labelled oligonucleotide probe 5'-CCATTCACCCAC-CAGCTCAGCTA-3' specific for the rat T cell receptor constant region (C β) elements (Chluba *et al.*, in preparation). α -chain RNA was detected by Northern blot hybridization with nick translated mouse complementary DNA probe²⁵.



specifically lyse ⁵¹Cr-labelled S1 target cells (Fig. 3). Moreover, as with the induction of anti-S1 proliferation, the cytotoxic effect of anti-S1 lymphocytes was unaffected by anti-MHC or anti-T cell antibodies (data not shown).

The neutralizing effect of anti-S1 cells on the S1 T line was also observed *in vivo*. Anti-S1 T cells were highly efficient in protecting syngeneic normal recipients from S1 line mediated tEAE. Full protection was seen when 6×10^6 anti-S1 cells were injected intravenously (i.v.) 1 day before, on the same day, or 1 day after injection of encephalitogenic S1 cells. Injection after delays of 48 h or more, however was not protective (Fig. 4). This particular kinetic behaviour is of interest for the pathogenesis of the CNS lesion in tEAE. We have shown previously that cells from a pathogenic T cell line break through the blood brain barrier within 24 h after transfer¹⁶. We have postulated that in the 72 h preceding clinical disease, these cells interact cytotoxicity with CNS cellular elements¹⁷ thus setting the stage for the full inflammatory reaction of EAE. Anti-S1 cells injected 48 h after transfer of pathogenic S1 cells may come too late to reverse the ongoing intrathecal response.

Our data document that rats recovered from tEAE possess T lymphocytes that give rise to CD8⁺ cell lines and that these cells effectively antagonize the encephalitogenic capacity of the MBP-specific CD4⁺ T cell line responsible for tEAE in the original donor. The T cell nature of the CD8⁺ lines described is unequivocally established both by their membrane phenotype, and by the demonstration of full-length mRNA for the T cell receptor α - and β -chain. The CD8⁺ lines thus behave like suppressor T cells *in vitro* as *in vivo*.

Cytotoxic CD8⁺ suppressor T lines have been described in the mouse¹⁸. These lines, in contrast to the ones described here, recognized protein antigen on APC. Our anti-S1 lines are strictly

specific for markers on S1 line cells, and completely ignore MBP. They thus resemble the CD8⁺ T cells induced *in vitro* against CD4⁺ T cell clones specific for either viral¹⁹, allo-²⁰ and self Ia antigens²¹. Interaction between both relevant T cell partners was described either as suppressive^{18,19} or cytolytic²¹. Two other interesting, and possibly related populations of suppressive CD8⁺ T cells have been isolated from lesions of lepra patients^{22,23}.

Which is the target antigen of the anti-S1 T lines? Anti-S1 T line cells were selectively responsive to determinants on S1 cells. Such complementary, 'clonotypic' T-T interactions have often

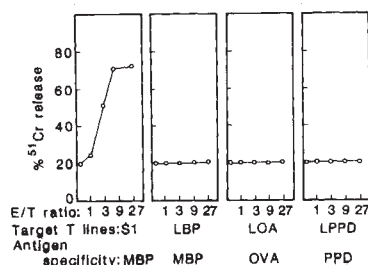


Fig. 3 Cytotoxicity of anti-S1 T line. Freshly activated blasts from CD4⁺ T cell lines were labelled with ⁵¹Cr (200 μ Ci ml⁻¹) for 1 h washed three times and used as targets in an isotope release assay. The target cells (T, 2×10^4) were incubated with graded numbers of anti-S1 effector cells (E) in 24-well plates for 20 h. The microplates were centrifuged, cell free supernatants were collected and radioactivity was determined in a γ -counter. ⁵¹Cr release was calculated (in %) by dividing c.p.m. obtained from cell-free supernatants by the total counts (supernatant plus sediment) of the individual cultures.

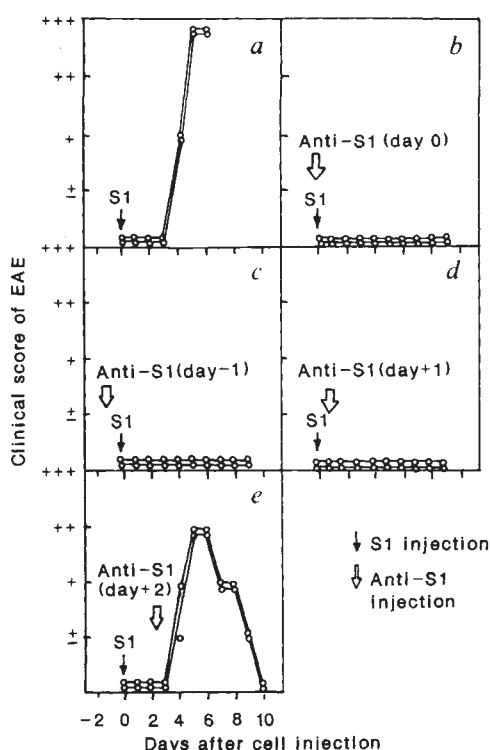


Fig. 4 *In vivo* protective activity of anti-S1 T line cells. Young adult Lewis rats were injected i.v. with 2×10^6 freshly activated encephalitogenic T cells from the line S1. Anti-S1 blasts (6×10^6) were injected i.v. at variant time points, as indicated. The recipient animals were weighed daily and scored for their clinical status: $\pm$, flaccid tail; +, hind limb weakness or paralysis; ++, total paralysis of hind limbs or tetraparesis; +++, moribund state and death.

been interpreted as anti-idiotypic, but other mechanisms should be considered as well. First there is the possibility, that anti-S1 cells might recognize MBP and/or MHC determinants on S1 cells. This is unlikely given their non-responsiveness to 'professional' MBP presenting cells. Second, the response could be directed against serum components adsorbed to the target cell surface. The clonotypic response pattern argues against this mechanism. Third, the S1/anti-S1 interaction could involve a 'trivial' rejection reaction against clonotypic neoantigens spontaneously appearing on S1 cells during their prolonged *in vitro* propagation. This is not probable, as S1 T cell lines can survive in Lewis hosts for more than 2 months (data not shown). Moreover, the finding that neither proliferative nor cytolytic responses can be blocked by antibodies against class I MHC determinants argues against a typical graft rejection response. The fourth possibility, an interaction between antigen receptors on both complementary T cell lines, that is, an idiotypic interaction *sensu strictu*, is thus possible, but clearly requires further experimental support. Antibodies against the T cells receptor complex may be of use in such studies.

Our present findings will be the basis for further experiments which may help us to learn, whether and how far $CD8^+$ suppressor/cytotoxic T cells have a role in physiological down-regulation of monophasic autoaggression in EAE, and perhaps, more generally, in the maintenance of immunological self tolerance.

We thank Mrs B. Goebel for preparing the manuscript. This Unit is supported by funds of the Hermann and Lilly Schilling-Stiftung.

Received 26 January; accepted 16 March 1988.

1. Alvord, E. C., Shaw, C. M., Hruby, S. & Kies, M. W. *Ann. N.Y. Acad. Sci.* **122**, 333-345 (1965).
2. Driscoll, B. F., Kies, M. W. & Alvord E. C. *J. Immun.* **128**, 635-638 (1982).

3. Ben-Nun, A., Wekerle, H. & Cohen, I. R. *Nature* **292**, 60-61 (1981).
4. McFarlin, D. E., Blanck, S. E. & Kibler, R. F. *J. Immun.* **113**, 712-715 (1974).
5. Willenborg, D. O. *J. Immun.* **123**, 1145-1150 (1979).
6. Ben-Nun, A. & Cohen, I. R. *J. Immun.* **128**, 1450-1457 (1982).
7. Adda, D. H., Beraud, E. & Depieds R. *Eur. J. Immun.* **7**, 620-623 (1977).
8. Bernard, C. C. A. *Clin. exp. Immun.* **29**, 100-109 (1977).
9. Swierkosz, J. E. & Swanborg, R. H. *J. Immun.* **115**, 631-633 (1975).
10. Welsh, A. M., Holda, J. H. & Swanborg, R. H. *J. Immun.* **125**, 186-189 (1980).
11. Lando, Z., Teitelbaum, D. & Arnon R. *J. Immun.* **126**, 1526-1528 (1981).
12. Schluesener, H. J. thesis, Würzburg Univ. (1985).
13. Asherson, G. L., Colizzi, V. & Zembala, M. A. *Rev. Immun.* **4**, 37-68 (1986).
14. Fierz, W., Endler, B., Reske, K., Wekerle, H. & Fontana A. *J. Immun.* **134**, 3785-3793 (1985).
15. Mason, D. W., Arthur, R. P., Dallman, M. J., Green, J. R., Spickett, G. P. & Thomas, M. L. *Immun. Rev.* **74**, 57-82 (1983).
16. Wekerle, H., Linington, C., Lassmann, H. & Meyermann, R. *Trends Neurosci.* **9**, 271-277 (1986).
17. Sun, D. & Wekerle, H. *Nature* **320**, 70-72 (1986).
18. Heuer, J., Brüner, K., Opalka, B. & Kölsch, E. *Nature* **296**, 456-458 (1982).
19. Lamb, J. R. & Feldman, M. *Nature* **300**, 456-458 (1982).
20. Mohaghehpour, N., Damle, N. K., Takada, S. & Engleman, E. G. *J. exp. Med.* **164**, 950-955 (1986).
21. Deusch, K., Moebius, U., Meyer zum Büschenfelde, K.-H. & Meuer, S. C. *Eur. J. Immun.* **16**, 1433-1438 (1986).
22. Modlin, R. L. *et al. Nature* **322**, 459-461 (1986).
23. Ottenhoff, T. H. M., Elferink, D. G., Klatzer, P. R. & deVries, R. R. P. *Nature* **322**, 462-464 (1986).
24. Hochgeschwender, U., Weltzien, H. U., Eichmann, K., Wallace, R. B. & Epplen, J. T. *Nature* **322**, 376-378 (1986).
25. Hochgeschwender, U., Simon, H.-G., Weltzien, H. U., Bartels, F., Becker, A. & Epplen, J. T. *Nature* **326**, 307-309 (1987).

A hypothetical model of the foreign antigen binding site of Class II histocompatibility molecules

Jerry H. Brown*, Theodore Jardetzky*, Mark A. Saper*, Boudjema Samraoui*, Pamela J. Bjorkman*†‡ & Don C. Wiley*§

* Department of Biochemistry and Molecular Biology, Harvard University, Howard Hughes Medical Institute, Harvard University, Cambridge, Massachusetts 02138, USA

† Department of Medical Microbiology, Stanford University, Stanford, California 94305, USA

Class II and class I histocompatibility molecules allow T cells to recognize 'processed' polypeptide antigens¹⁻⁵. The two polypeptide chains of class II molecules, α and β , are each composed of two domains (for review see ref. 6); the N-terminal domains of each, α_1 and β_1 , are highly polymorphic⁶⁻¹⁰ and appear responsible for binding peptides at what appears to be a single site¹¹⁻¹³ and for being recognized by MHC-restricted antigen-specific T cells^{14,15}. Recently, the three-dimensional structure of the foreign antigen binding site of a class I histocompatibility antigen has been described^{16,17}. Because a crystal structure of a class II molecule is not available, we have sought evidence in class II molecules for the structural features observed in the class I binding site by comparing the patterns of conserved and polymorphic residues of twenty-six class I and fifty-four class II amino acid sequences. The hypothetical class II foreign-antigen binding site we present is consistent with mutation experiments¹⁸⁻²⁸ and provides a structural framework for proposing peptide binding models to help understand recent peptide binding data^{11,29-33}.

An overall similarity in structures of class I and class II molecules is suggested by a similarity in domain and genomic organization⁶, by high similarity in the C-terminal domain sequences³⁴, and by the observation that some T cells specific for either class of molecule use the same receptor^{35,36}. Furthermore, the close association of N-terminal residues of the α_1 and β_1 domains in the class II intermolecular dimer, deduced from hemi-exon shuffling experiments^{37,38}, is consistent with the close association of N-terminal residues of the class I α_1 and α_2 domains in the 'intramolecular' dimer observed in the crystal

‡ Present address: Department of Medical Microbiology, Stanford University, Stanford, California 94305, USA.

§ To whom correspondence should be addressed.

structure¹⁶. By imagining an attachment of the class I α_1 domain to β_2 -microglobulin, a four domain model of class II molecules can be produced from the class I structure¹⁶. Like the class I structure (Fig. 2a), such a class II molecule would have a cleft between the C-terminal α -helices of its polymorphic α_1 and β_1 domains with the bottom of the cleft formed by the N-terminal β -strands of each domain¹⁷.

Pairwise sequence comparisons of class II α_1 domains with the polymorphic domains of class I (α_1 and α_2) revealed no significant sequence similarity, whereas comparisons of class II β_1 domains revealed weakly significant similarity with both the α_1 and α_2 domains of class I³⁹⁻⁴¹. The choice to align class II α_1 to class I α_1 and class II β_1 to class I α_2 is suggested by the glycosylation site (Asn, X, Ser/Thr) at residue 86 (class II residues in the text have been renumbered to class I HLA-A2 numbering to facilitate comparisons to the class I three-dimensional structure, see legend to Fig. 1), which is completely conserved in all class I and class II α_1 sequences, and by the disulphide-linked cysteines (101 and 164) found in all class I α_2 and class II β_1 sequences⁶ (Fig. 1). This choice has been supported both by similarities in the respective genomic structures⁴² and by similarities in the alteration of T-cell recognition by a pattern of mutation at 152, 155, and 156 in both class I α_2 (bm1 mutant) sequences⁴³ and class II β_1 (bm12 mutant) sequences^{28,44}.

Evidence for the presence of homologous α -helices in class I and class II structures is found in the pattern of conserved amino acids. In the α -helical regions of all class I sequences these occur every third or fourth residue⁴⁵ (conserved α_1 residues 68, 72, 75, 78, 84, conserved α_2 residues 148, 153, 154, 157, 158, 161, 164, 165, 168; solid columns in Fig. 1). These residues form one face of the α -helices in class I α_1 and α_2 domains¹⁷ (Fig. 2a) and their positions are also conserved in class II α_1 and β_1 sequences (Fig. 1). Interspersed between the conserved residues on the α -helices of the class I structure are many of the most polymorphic residues¹⁷. Again, a similar pattern of polymorphic residues is also found in class II sequences [α_1 : 74, 79, 80, 81; β_1 : 152, 155, 156, 159, 163 (class I numbering, see Fig. 1)]. Structural similarity in the helical regions is further supported by the observation of a completely conserved i, i+4 helix-stabilizing salt bridge (Arg157–Acid161, see Fig. 2b) and the conserved Trp147 in the class I α_2 and class II β_1 domains, and the completely conserved Leu78 in class I and class II α_1 domains (Fig. 1).

In the β -strand region (N-terminal half of the sequences in Fig. 1), the distribution of polymorphic residues in class II sequences often match those in class I sequences. In Fig. 1, the most polymorphic positions in each strand in the class I α_1 domain are aligned to the polymorphic residues in the corresponding regions of class II sequences (9 in S1, 24 in S2, 32 in S3, 45 in S4; cross-hatched columns Fig. 1, see legend for a fuller explanation of alignment, particularly in S1). A similar alignment of polymorphic residues in class I α_2 and class II β_1 (95, 97, 99 in S1; 114, 116 in S2; Fig. 1) is provided by matching the conserved cysteine 101. These polymorphic residues form the 'bottom' of the cleft in the class I structure (Fig. 2a). The striking 'every other one' periodicity (95, 97, 99, and 114, 116) of the polymorphic residues is that expected for β -strand residues where every other side chain faces to one side of a sheet (see legend, Fig. 1).

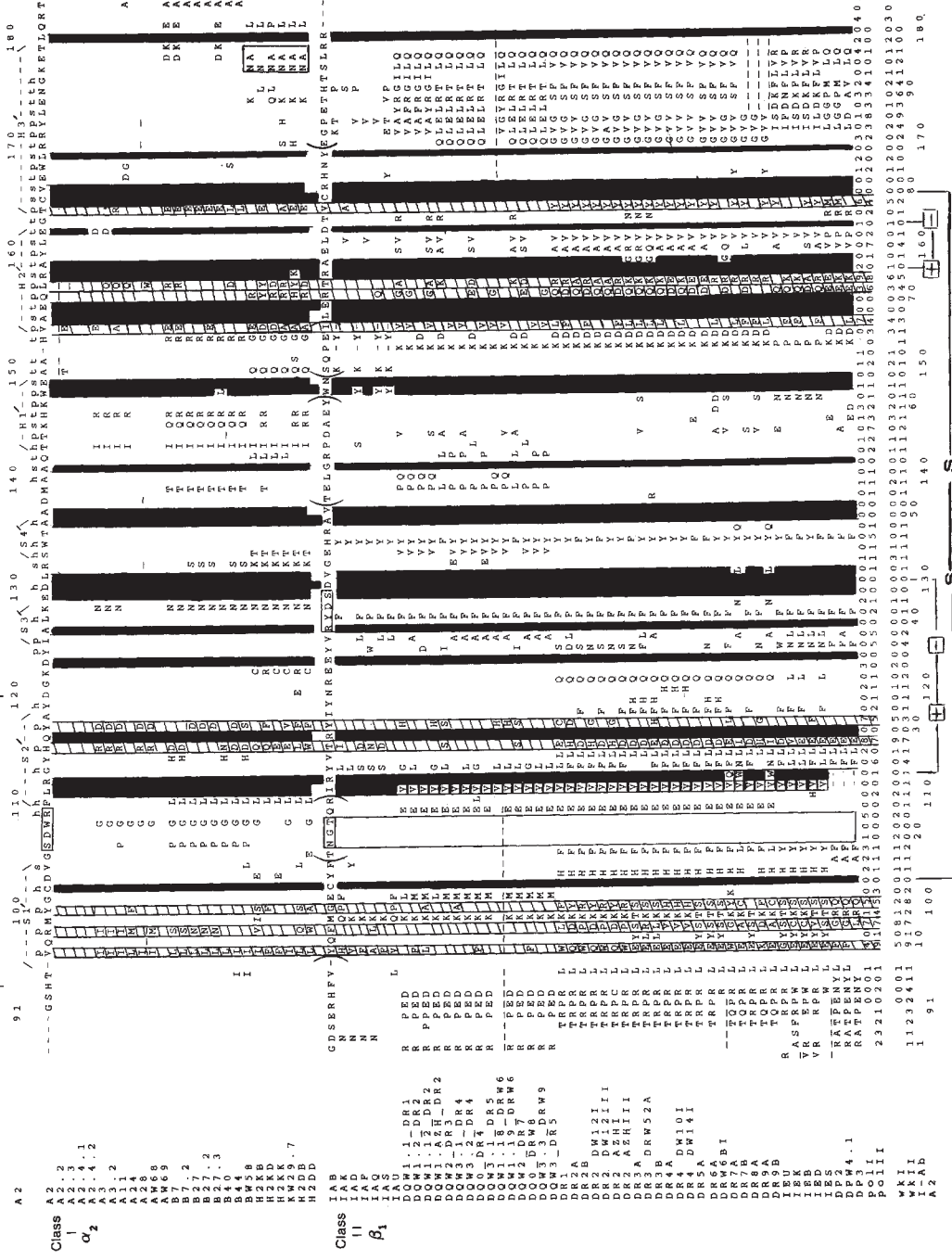
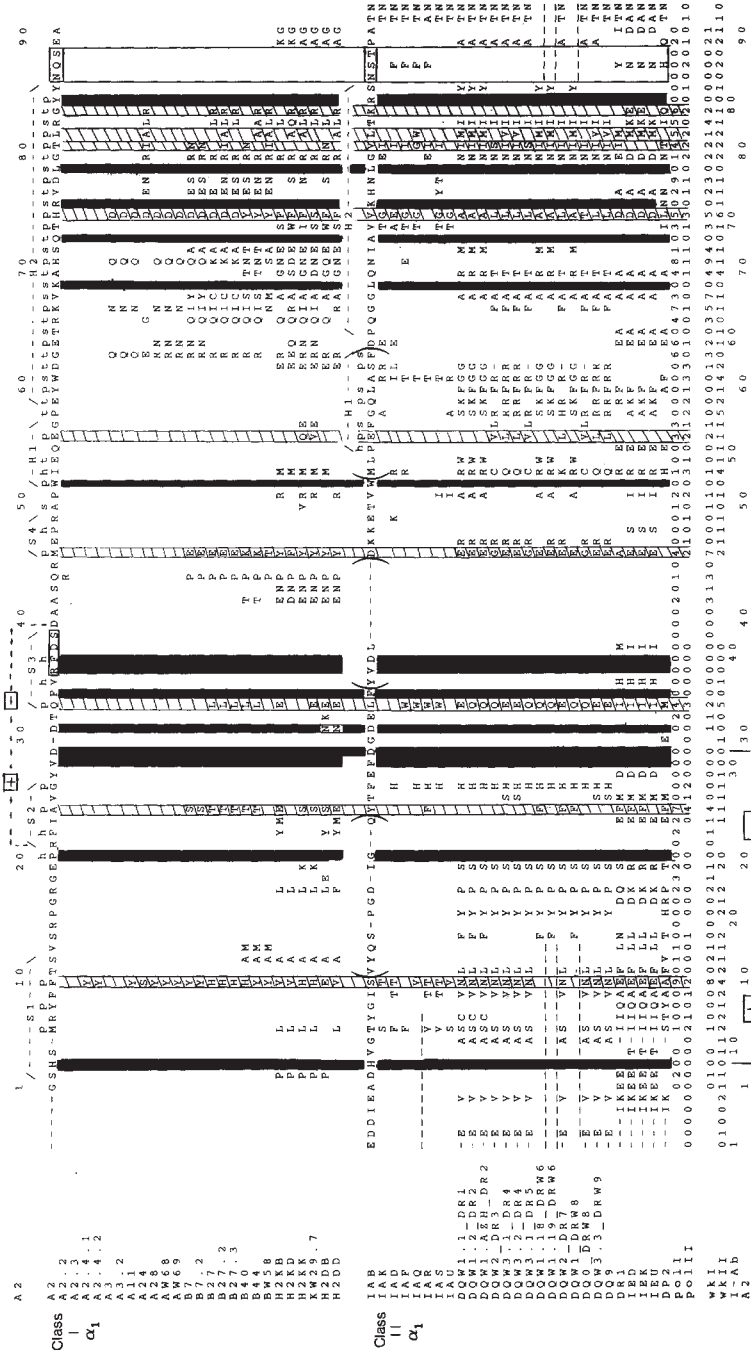
Structural homology in the β -strand region is further supported by the observation that the residues making up the two salt-bridges His3–Asp29 and Arg111–Asp129, which bridge across the open end of the two β -strand 'hairpins' formed by S1 and S2 and by S2' and S3' (Fig. 2b), are conserved in all class I and class II sequences (Fig. 1b), and are the same distance from the first polymorphic residues of their β -strands in both class I and class II [in S1, I-A and DQ are aligned to other class II and class I sequences by introducing a β -bulge⁴⁶ at Val4 (Gly bulges out) which results in I-A polymorphic position 5 (residue 10 in I-A numbering), as well as the aligned polymorphic position 9 pointing up into the proposed site]. Interestingly, the pseudo-two-fold symmetric locations His93–Asp119 and Arg21–Asp39 (dotted lines in Fig. 2b) are conserved salt links in all class I sequences, but not class II sequences.

The regions between the aligned secondary structure elements are consistent with loop structures. Class I and class II share common amino acid residues in these regions (Fig. 2b). Insertions and deletions in Class II sequences relative to class I sequences occur in or at the boundary of these loops (Fig. 1). In class II, a glycosylation site at the loop containing residues 105–108 and a seven residue deletion of the loop at residues 35–38 (underneath a suggested shift in the bend of the α_1 α -helix described in the legend to Fig. 3) eliminate the sequences SDWR and RFDS, respectively, previously noted as potential CD8 interaction sites in class I⁴⁷ (see Fig. 2b).

The alignment presented in Fig. 1 allows us to propose a hypothetical structure for the foreign antigen recognition site

Fig. 1 (Opposite) Alignment of class II α_1 and β_1 domains to class I α_1 and α_2 domains. Because of lack of statistically significant pairwise homology between class II α_1 and class I α_1 ³⁹, this alignment is based on the matching of positions polymorphic in both class I and class II (hatched) and of positions conserved in both classes (solid). (Also included are positions 30, 75, 111, and 147, which are conserved in at least 90% of the sequences, and position 110, which is either Leu, Ile or Val in all sequences.) This analysis agrees with a previous pairwise alignment of DR2 β_1 and HLA-B7 α_2 ⁴¹. The sequence alignments include one letter code (deviations from A2 or I-A^b sequences), blanks (identity to HLA-A2 or I-A^b), dashes (deletions), or underscores (undetermined residues). Sequence numbers both of HLA-A2 (top) and of I-A^b (bottom) are shown. The approximate secondary structural boundaries observed in HLA-A2 (ref. 16) are indicated by S1 (first β -strand of α_1), S2 (second β -strand), H1 (first helix), S1' (first β -strand of α_2), etcetera. Regions towards which HLA-A2 amino acid side chains point are described by p (processed antigen binding site), h (helical region), s (strand region), t ('T cell')¹⁷. Charge pair (+–), and disulphide (S–S) interactions observed in HLA-A2 and conserved in the class I and the aligned class II sequences (see also Fig. 2) are shown as solid connections. Dashed connections indicate conservation of the charge pair through a third (but not a fourth) domain (see text). The alignments within the parentheses are not constrained by the structural evidence presented. WkI and wkII are normalized polymorphism values⁵⁵ of each position in the twenty-six class I and the fifty-four class II sequences, respectively. PolI and polII are normalized modified polymorphism values defined as: $\ln [I_i \{VAR_i * SDHYD_i + VAR_i\}]$ where i is an isotype, $VAR(i)$ is a position's variability index⁵⁵, within the isotype, and $SDHYD_i$, which de-emphasizes chance hydrophobic diversity⁵⁶, is the standard deviation of the hydrophobicity of the position's residues⁵⁷ within the isotype. The multiplication of the individual isotypes' modified polymorphism values results in the position's overall class polymorphism value (pol) to be zero only if the position is conserved within each isotype, whereas $\text{pol} = 9$ only if the position is polymorphic in each of the isotypes. Polymorphism in class I is highly correlated to proximity of the side chain to the processed antigen binding site¹⁷; 14 out of the 20 most polymorphic (wk) class I residues face into the site. This correlation using modified polymorphism values (pol) is even slightly higher, 16/20 and justifies the use of this measure to aid in the alignment of site residues. Those positions with modified polymorphism values (pol) among an individual domain's top 20 are considered polymorphic in this report. Structural considerations were used in the alignment of S1 to resolve the ambiguity of predictions based on polymorphism values alone. [In S1, position 5 has the same pol II value as position 9. The wk-II value (averaged over all isotypes) is greater for 9 than 5 but position 5 is more polymorphic than 9 if the I-A isotype is considered alone. However, the three side chains of S1 (positions 5, 7, 9) form the bottom of the cleft in the class I structure and only the alignment shown places both polymorphic residues (5, 9) in the site (Fig. 3a). This choice simultaneously preserves the distance between the residues and a conserved structural feature provided by His3 (see text and Fig. 2b).] In class I, the four isotypes used in the polymorphism calculations are HLA-A*8, HLA-B*8, H2K*59, H2D*59. In class II, the five isotypes used are DR(1, 2a, 2b, 3a, 3b, 4a, 5a, 6a, 7a, 7b, 8a, 9a, 9b, others⁴⁹), I-E⁶¹, I-A (α_1 ⁶², β_1 ⁶³), DQ (w3-dr5⁶⁴, 9⁶⁵, all others⁴⁹) and DP⁶⁶. An enlarged copy of this figure can be obtained from the authors or by Xerographic enlargement.

LETTERS TO NATURE



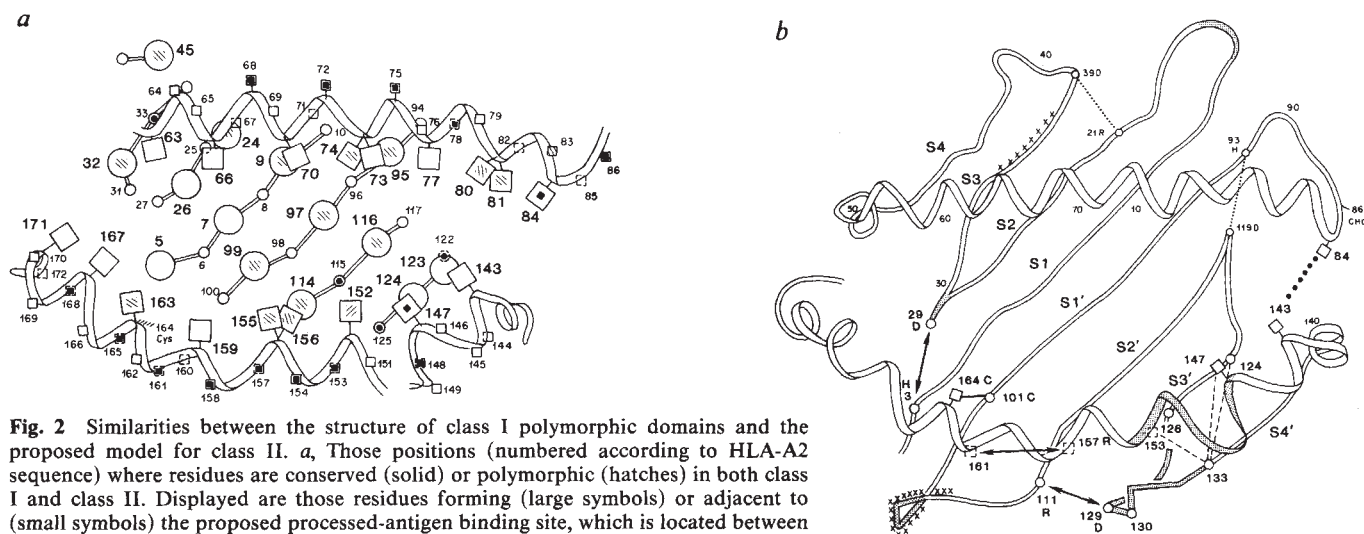


Fig. 2 Similarities between the structure of class I polymorphic domains and the proposed model for class II. *a*, Those positions (numbered according to HLA-A2 sequence) where residues are conserved (solid) or polymorphic (hatches) in both class I and class II. Displayed are those residues forming (large symbols) or adjacent to (small symbols) the proposed processed-antigen binding site, which is located between the α -helices (squares) and above the β -strands (circles) in the class I structure¹⁷.

Positions of residues conserved in both MHC classes generally face away from the site, while those polymorphic in both classes face into the site. In both class I and class II molecules, different isotypes have different distributions of polymorphic residues⁶⁷. Nevertheless, the sequence similarity among isotypes^{6,7,58,59} argues that the structural features of the site are essentially the same in different isotypes and validates the construction of a single overall class II model. H1 is not shown here. The break at the kink between helices at 149 indicates a site of insertions and deletions. *b*, Intramolecular interactions observed in the HLA-A2 structure¹⁶ and conserved in all class I and class II sequences (Fig. 1). Three salt bridges (solid lines bounded by arrows), whose residues are conserved in all class I and class II sequences (111 is Arg in all but three sequences, 157 is Glu or Asp), along with alignments of other conserved residues and of polymorphic residues (see Fig. 2*a* and text), argue that class I and class II have homologous β -strands and α -helices. Evidence for the similar positioning of the β -strands relative to the α -helices includes the disulphide bridges (101 to 164) and the hydrophobic cluster (dashed lines 124, 133, 147, 153) conserved in all class I and class II sequences. Bold dots at 84, 143 indicate the position of the Tyr-Thr hydrogen bond conserved in class I, and a potential salt bridge, semi-conserved in class II and implicated in resistance to IDDM (see text)^{49,50}. Xs mark class I RFDS-like sequences⁴⁷ (see text). Shaded loops contain similar residues in class I and class II: S1-S2 (Pro and Gly), S2-S3 (two acids), S1'-S2' (class I Pro, Gly; class II Asn, Gly), S3'-S4' (126, 130, 133 conserved hydrophobics), H1'-H2' (conserved 147 Trp, 154 Glu). Deletions and insertions in class II sequences relative to class I occur in loops S1-S2, S2-S3, S3-S4, H1'-H2', and at the N- and C-terminals of both α_1 and β_1 domains. CHO (residue 86) is the common carbohydrate attachment site. The second class II CHO site (105-107) is found in the shaded loop after 103.

Table 1 The effects of class II single amino acid mutations on monoclonal antibody binding and T-cell activation

Residue numbering		Substitution	Monoclonal antibody	T cells		Reference	Location on hypothetical class II structure
HLA-A2	Ia			allo	ag		
79	I-A ^k _{α}	75 E→K	+(4/5)	-(0/2)	+(2/17)	18, 19	H2, solvent-accessible, pointing up
95	I-A ^b _{β}	9 Y→H	-(0/12)	+(2/7)	-(0/3)	20	S1', pointing up into helix
95	I-A ^b _{β}	9 H→V	-(0/8)	ND	ND	21	S1', pointing up into helix
98	I-A ^b _{β}	12 Q→K	-(0/8)	ND	ND	21	S1', pointing down
99	I-A ^b _{β}	13 G→P	-(0/12)	+(1/7)	+(1/3)	20	S1', pointing up in crevice
99	I-A ^k _{β}	13 P→G	-(0/8)	ND	ND	21	S1', pointing up into site
100	I-A ^k _{β}	14 F→E	-(0/8)	ND	ND	21	S1', pointing down
103	I-A ^k _{β}	17 F→Y	-(0/8)	ND	ND	21	S1'-S2'
114	I-E ^k _{β}	29 V→E	ND	ND	+(5/7)	22	S2', pointing up into site
114	I-A ^k _{β}	28 I→T	-(0/8)	ND	ND	21	S2', pointing up into site
126	I-A ^k _{β}	40 F→Y	-(0/8)	ND	ND	21	S3', pointing up into helix (see caption)
134	I-E ^k _{β}	49 R→H	+(4/16)	-(0/2)	-(0/17)	23, 24	S4', solvent-accessible, pointing down towards the region of the lower domains
134	I-A ^k _{β}	48 R→C	+(2/7)	-(0/2)	-(0/15)	*	S4', solvent-accessible, pointing down
145	I-A ^k _{β}	59 E→D	+(3/10)	ND	ND	25	H1', solvent-accessible, pointing up
149	I-A ^b _{β}	63 K→S	+(1/8)	ND	ND	21	H1'-H2', kink between helices
150	I-A ^b _{β}	64 Q→P	+(7/10)	-(0/1)	+(7/15)	25, 26	H1'-H2', kink between helices
150	I-A ^b _{β}	64 Q→R	+(1/10)	-(0/1)	+(2/15)	25, 26	H1'-H2', kink between helices
154	I-A ^b _{β}	67 E→K	+(1/3)	ND	+(1/2)	27	H2', solvent-accessible, pointing up
152	I-A ^b _{β}	67 I→F	-(0/6)	ND	+(5/6)	28	H2', into site
155	I-A ^b _{β}	68 R→Q	+(4/10)	+(1/1)	+(11/15)	25, 26	H2', up and into top part of the site
155	I-A ^b _{β}	70 R→Q	+(4/6)	ND	+(6/6)	28	H2', up and into the top part of the site
156	I-A ^b _{β}	71 T→K	-(NC)	ND	+(5/5)	28	H2', into site
160	I-A ^k _{β}	73 L→V	-(0/7)	ND	ND	21	H2', down towards strand
163	I-A ^b _{β}	76 V→A	-(0/8)	ND	ND	21	H3', into site
170	I-A ^b _{β}	83 K→G	-(0/8)	ND	ND	21	H3', up (?)
171	I-A ^k _{β}	84 T→P	-(0/8)	ND	ND	21	H3', into site (?)
174	I-A ^k _{β}	87 P→S	-(0/8)	ND	ND	21	H3', (?)

(x/y) Indicates a loss of reactivity with x parent Ia monoclonal antibodies, or x allospecific, or foreign antigen-restricted (ag) T cells out of a total of y antibodies/T-cell clones examined, as a result of the single substitution indicated. +, (x > 0) indicates that the residue is involved in monoclonal antibody or T-cell recognition. -, indicates that the residue may not be involved in monoclonal antibody or T-cell recognition. The mutation at 126, which does not cause loss of binding by any parent I-A^k-reactive antibodies, does cause a gain in binding by ('non-parent') I-A^b-reactive antibodies²¹. ND, no data; NC, not clear; *, Glimcher *et al.*, personal communication. The location of the altered side chains in the hypothetical class II model are consistent with these effects (see text).

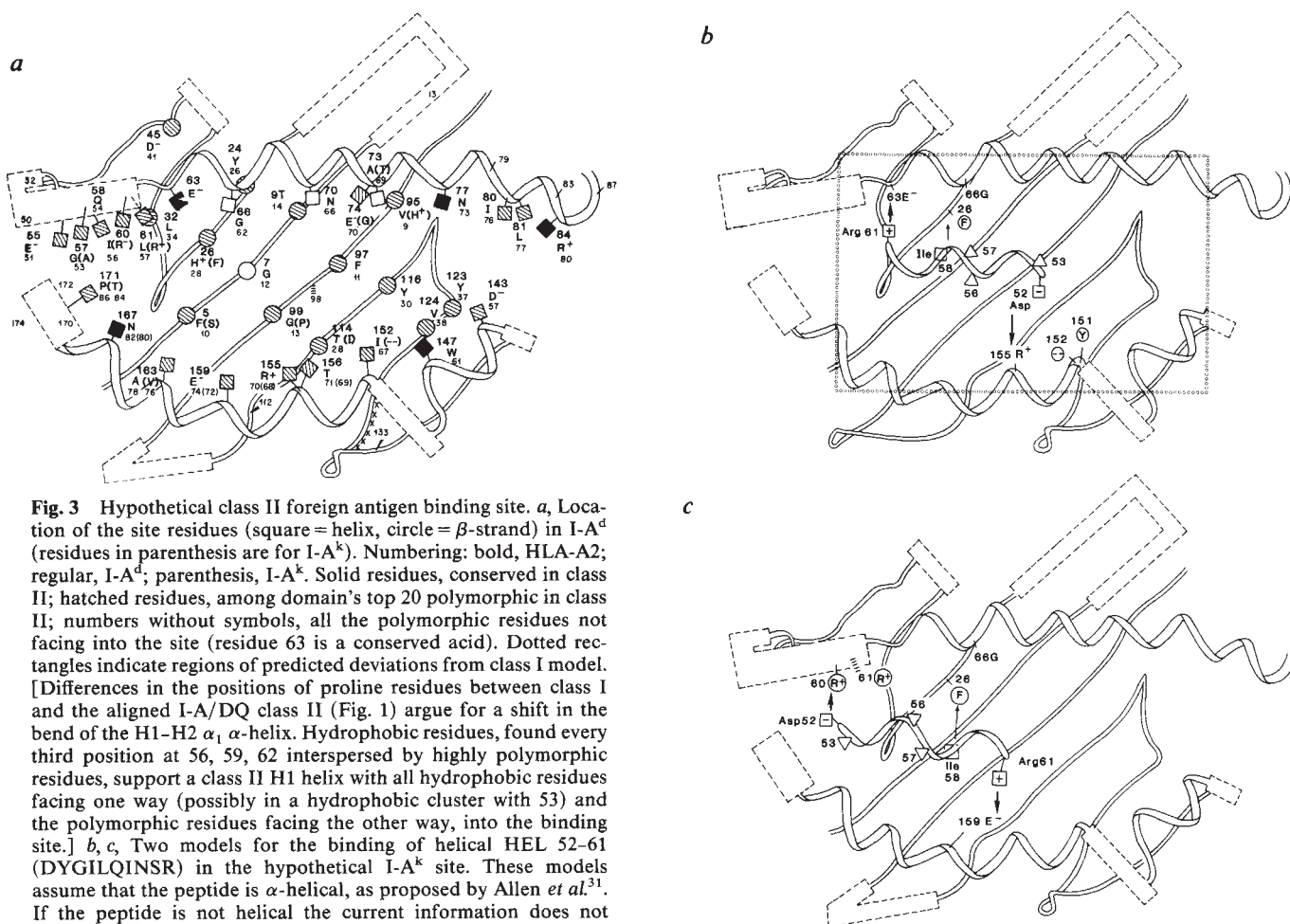


Fig. 3 Hypothetical class II foreign antigen binding site. *a*, Location of the site residues (square = helix, circle = β -strand) in I-A^d (residues in parenthesis are for I-A^k). Numbering: bold, HLA-A2; regular, I-A^d; parenthesis, I-A^k. Solid residues, conserved in class II; hatched residues, among domain's top 20 polymorphic in class II; numbers without symbols, all the polymorphic residues not facing into the site (residue 63 is a conserved acid). Dotted rectangles indicate regions of predicted deviations from class I model. [Differences in the positions of proline residues between class I and the aligned I-A/DQ class II (Fig. 1) argue for a shift in the bend of the H1-H2 α_1 -helix. Hydrophobic residues, found every third position at 56, 59, 62 interspersed by highly polymorphic residues, support a class II H1 helix with all hydrophobic residues facing one way (possibly in a hydrophobic cluster with 53) and the polymorphic residues facing the other way, into the binding site.] *b*, *c*, Two models for the binding of helical HEL 52-61 (DYGILQINSR) in the hypothetical I-A^k site. These models assume that the peptide is α -helical, as proposed by Allen *et al.*³¹. If the peptide is not helical the current information does not sufficiently limit the degrees of freedom to allow a model of the complex to be proposed. Squares represent the peptide residues proposed³¹ to be involved in 'MHC-contact'; triangles are the peptide's proposed 'T-cell-contact' residues³¹. I-A^k residues are indicated using the single letter code. Bold lines indicate potential salt bridges (see text). Circled residues are different in the non-responder I-A^d allele and may be responsible for lack of binding of the peptide to I-A^d. [For example Phe26 which is near peptide Ile58 is His in I-A^d, introducing a polar residue into a hydrophobic contact. And positions corresponding to 151 and 152 (HLA-A2) change from Tyr in I-A^k to Glu-Ile in I-A^d (the largest insertion in the site). In I-A^d the glutamic acid may form a salt bridge with Arg155 (151 and 155 are within hydrogen bonding distance in HLA-A2); this might eliminate Arg 155 as a potential partner to salt bridge to the peptide.] In the context of this hypothetical binding model, other non-responder alleles could result from other substitutions in the vicinity of the peptide.

of class II molecules based (Fig. 3*a*) on structural homology with the class I site¹⁷. The distribution of polymorphic residues (crosshatched, Fig. 3*a*) in the site is consistent with the conclusions that some of the polymorphic residues (for example, those on the bottom of the site) probably control interaction with antigen^{22,48} whereas others control allele-related recognition by T cells^{22,48}. It is also consistent with studies of natural²⁸ and site-directed mutants¹⁸⁻²⁷ of class II molecules (Table 1). Residues which are monoclonal antibody epitopes (79, 134, 145, 149, 150, 154, 155, Fig. 3*a*) protrude into solvent and residues implicated in antigen-restricted T-cell recognition face into the site (95, 99, 114, 152) or upwards (presumably toward the T cell) (79, 150, 154, 155, 156, Fig. 3*a*). Aspartic acid at position 143 in DQ (57 in DQ β numbering), which provides almost complete resistance to insulin-dependent diabetes (IDDM)^{49,50}, is predicted to form a salt bridge with Arg84 at one end of the site.

The hypothetical model provides a framework for generating testable models for peptide binding. There is currently no consensus as to the conformation that peptides adopt when bound³¹⁻³³, or even whether or not different peptides adopt similar backbone conformations upon binding. The size, shape, and residue composition of the site in HLA-A2 indicate that it

has space to accommodate an α -helical or extended conformation, or a mixture of both, and therefore leaves open many possibilities¹⁷. In cases where the conformation of a bound peptide can be proposed from other data or where contacts between the peptide and specific residues on the histocompatibility antigens can be identified, the number of degrees of freedom may be reduced enough to allow specific models of the complex to be proposed. The hen egg-white lysozyme peptide binding to I-A^k is such a case³¹. HEL (46-61) binds to I-A^k but not to I-A^d^{13,29}. In the 10-mer HEL(52-61), three 'MHC-contact' and three 'T-cell contact' residues segregate on the opposite sides of the bound peptide if it binds as an α -helix³¹. Because Asp52 is 2.5 turns from Arg61 on an ideal helix, it is possible that one residue contacts the helix of the α_1 domain and the other contacts the helix of the β_1 domain¹⁷. By sliding this 2.5 turn helix in the site, two ways are found which both stabilize the charged peptide residues by oppositely charged I-A^k helix residues and keep the 'T-cell contact' residues pointing up (Fig. 3*b,c*). Both models also result in the third defined I-A^k 'contact residue', Ile58, contacting Phe26 from the β -sheet and Gly66 from the helix. Differences between I-A^k and I-A^d at residues 26 and/or 151 could account for the absence of binding to I-A^d (see legend to Fig. 3*b,c*). The dotted rectangle

on Fig. 3b represents the area of an Fab-antigen interface⁵¹⁻⁵³, to indicate that the T-cell receptor, if it binds like an Fab with which it has sequence homology⁵⁴, could recognize both the Ia molecule and the peptide simultaneously.

In two other studies of peptide-MHC interaction [OVA (323-339)³², PCC (93-104)³³] no simple conformation could be deduced, but the data did not favour an α -helix. These peptides were shown to contain many positions with both MHC and T-cell contact residue characteristics. Such dual character could be expected for some peptide residues, given the structure of the binding site, but it could also arise from reorientation of single substituted peptides within the binding site, thereby obscuring any structural pattern. However, if the actual bound state of these peptides is as conformationally complex as these studies imply, then more detailed structural restrictions on peptide binding are a prerequisite to modelling.

Whereas the above studies locate those peptide residues that contact the MHC molecule, a recent study²² has implicated I-E^k residue Val114 in contacting the PCC peptide and not the T cell. This is consistent with the model in Fig. 3a, since position 114 points up to the site from the β -sheet region and is probably inaccessible to direct interaction with the T-cell receptor¹⁷. A study showing Lys103 of PCC to contact only the MHC and not the T cell³³, suggests that PCC 103 may contact the β -sheet region of the site.

The hypothetical model for a class II foreign antigen binding site may be useful for formulating testable hypotheses about foreign antigen and T-cell binding. We note, however, that significant shifts in the direction (up to 30°) and location (up to 7 Å) of α -helices and β -strands (20°, 2 Å) have been observed in the X-ray structures of the very closely related members of both the globin and the IgG families^{56,68}. Therefore any hypothetical model derived from even closely related sequences may differ from the true structure in important details and should be thought of only as an imperfect guide for experiments.

Boudjema Samraoui thanks the Algerian Ministère de l'Enseignement et de la Recherche Scientifique for a postdoctoral leave. Pamela J. Bjorkman held an American Cancer Society postdoctoral fellowship during part of the work. The research was supported by the NIH and the Howard Hughes Medical Institute.

Received 19 January; accepted 18 March 1988.

- Benacerraf, B. *J. Immun.* **120**, 1809-1812 (1978).
- Rock, K. L. & Benacerraf, B. *J. exp. Med.* **159**, 1238-1252 (1984).
- Ziegler, H. K. & Unanue, E. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 175-178 (1982).
- Shimonkevitz, R., Kappler, J., Marrack, P. & Grey, H. *J. exp. Med.* **158**, 303-316 (1983).
- Townsend, A. R. M., Gotch, F. M. & Davey, J. *Cell* **42**, 457-468 (1985).
- Kaufman, J. F., Aufray, C., Korman, A. J., Shackelford, D. A. & Strominger, J. L. *Cell* **36**, 1-13 (1984).
- Benoist, C. O., Mathis, D. J., Kanter, M. R., Williams II, V. E. & McDevitt, H. O. *Cell* **34**, 169-177 (1983).
- Choi, E., McIntyre, K., Germain, R. N. & Seidman, J. G. *Science* **221**, 283-286 (1983).
- Larhammer, D. *et al. Cell* **34**, 179-188 (1983).
- Schenning, L. *et al. EMBO J.* **3**, 447-452 (1984).
- Guillet, J.-G. *et al. Science* **235**, 865-870 (1987).
- Buus, S., Sette, A., Colon, S. M., Miles, C. & Grey, H. M. *Science* **235**, 1353-1358 (1987).
- Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E. & Unanue, E. R. *Nature* **317**, 359-360 (1985).
- Folsom, V., Gay, D. & Tonegawa, S. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1678-1682 (1985).
- Germain, R. N. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 2940-2944 (1985).
- Bjorkman, P. J. *et al. Nature* **329**, 506-512 (1987).
- Bjorkman, P. J. *et al. Nature* **329**, 512-518 (1987).
- Landais, D. *et al. Cell* **47**, 173-181 (1986).
- Griffith, I. J., Choi, E. M. & Glimcher, L. H. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1090-1093 (1987).
- Cohn, L. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 747-751 (1986).
- Buerstedde, J.-M. *et al. J. exp. Med.* (in the press).
- Ronchese, F., Schwartz, R. H. & Germain, R. N. *Nature* **329**, 254-256 (1987).
- Quill, H., Schwartz, R. H. & Glimcher, L. H. *J. Immun.* **136**, 3351-3359 (1986).
- Griffith, I. J., Carland, F. M. & Glimcher, L. H. *J. Immun.* **138**, 4480-4483 (1987).
- Beck, B. N. *et al. J. exp. Med.* **166**, 433-443 (1987).
- Beck, B. N., Glimcher, L. H., Nilson, A. E., Pierres, M. & McKean, D. J. *J. Immun.* **133**, 3176-3181 (1984).
- Brown, M. A., Glimcher, L. H., Nielsen, E. A., Paul, W. E. & Germain, R. N. *Science* **231**, 255-258 (1986).
- Ronchese, F., Brown, M. A. & Germain, R. N. *J. Immun.* **139**, 629-638 (1987).
- Babbitt, B. P., Matsueda, G., Haber, E., Unanue, E. R. & Allen, P. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4509-4513 (1986).
- Buus, S., Sette, A., Colon, S. M., Jenis, D. M. & Grey, H. M. *Cell* **47**, 1071-1077 (1986).

- Allen, P. M. *et al. Nature* **327**, 713-715 (1987).
- Sette, A., Buus, S., Colon, S., Smith, J. A., Miles, C. & Grey, H. M. *Nature* **328**, 395-399 (1987).
- Fox, B. S. *et al. J. Immun.* **139**, 1578-1588 (1987).
- Travers, P., Blundell, T. L., Sternberg, M. J. E. & Bodmer, W. *Nature* **310**, 235-238 (1984).
- Rupp, F., Acha-Orbea, H., Hengartner, H., Zinkernagel, R. & Joho, R. *Nature* **315**, 425-427 (1985).
- Marrack, P. & Kappler, F. *Adv. Immun.* **38**, 1-30 (1986).
- Braunstein, N. S. & Germain, R. N. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2921-2925 (1987).
- Sant, A. J., Braunstein, N. S. & Germain, R. N. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8065-8069 (1987).
- Korman, A. J., Aufray, C., Schamboeck, A. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6013-6017 (1982).
- Larhammer, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3687-3691 (1982).
- Travers, P., thesis, Univ. London (1984).
- Hood, L., Steinmetz, M. & Malissen, B. *A. Rev. Immun.* **1**, 529-568 (1983).
- Nathenson, S. G., Geliebter, J., Pfaffenbach, G. M. & Zeff, R. A. *A. Rev. Immun.* **4**, 471-502 (1986).
- Michaelides, M., Sandrin, M., Morgan, G., McKenzie, I. F. C., Ashman, R. & Melvold, R. *W. J. exp. Med.* **153**, 464-469 (1981).
- Gussow, D. *et al. Immunogenetics* **25**, 313-322 (1987).
- Richardson, J. S., Getzoff, E. D. & Richardson, D. C. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2574-2578 (1978).
- Aufray, C. & Novotny, J. *Hum. Immun.* **15**, 381-390 (1986).
- Heber-Katz, E., Hansburg, D. & Schwartz, R. H. *J. Molec. cell. Immun.* **1**, 3-14 (1983).
- Todd, J. A., Bell, J. I. & McDevitt, H. O. *Nature* **329**, 599-604 (1987).
- Horn, G. T., Bugawan, T. L., Long, C. M. & Erlich, H. A. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Amit, A. G., Mariuzza, R. A., Phillips, S. E. V. & Poljak, R. J. *Science* **233**, 747-753 (1986).
- Colman, P. M. *et al. Nature* **326**, 358-363 (1987).
- Sheriff, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 8075-8079 (1987).
- Hedrick, S. M., Nielsen, E. A., Kavalier, J., Cohen, D. I. & Davis, M. M. *Nature* **308**, 253-258 (1984).
- Wu, T. T. & Kabat, E. A. *J. exp. Med.* **132**, 211-250 (1970).
- Lesk, A. M. & Chothia, C. *J. molec. Biol.* **136**, 225-270 (1980).
- Kuntz, I. D. *J. Am. chem. Soc.* **93**, 516-518 (1971).
- Cowan, E. P., Jelachich, M. L., Biddison, W. E. & Coligan, J. E. *Immunogenetics* **25**, 241-250 (1987).
- Immunology Today*, Centre-page Diagram, Ref. No. 13 (Elsevier, Cambridge, U.K.).
- Bell, J. I. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 6234-6238 (1987).
- Ayane, M., Mengle-Gaw, L., McDevitt, H. O., Benoist, C. & Mathis, D. *J. Immun.* **137**, 948-951 (1986).
- Landais, D., Matthes, H., Benoist, C. & Mathis, D. *Proc. natn. Acad. Sci. U.S.A.* **82**, 2930-2934 (1985).
- Estess, P., Begovich, A. B., Koo, M., Jones, P. P. & McDevitt, H. O. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3594-3598 (1986).
- Schiffenbauer, J. *et al. J. Immun.* **139**, 228-233 (1987).
- Jonsson, A. *et al. J. biol. Chem.* **262**, 8767-8777 (1987).
- Korman, A. J. *et al. Immun. Rev.* **85**, 45-86 (1985).
- Kappes, D. & Strominger, J. L. *A. Rev. Biochem.* **57** (in the press).
- Lesk, A. M. & Chothia, C. *J. molec. Biol.* **160**, 325-342 (1982).

Identification of a second human retinoic acid receptor

Nigel Brand*, Martin Petkovich*, Andrée Krust*,
Pierre Chambon*†, Hugues de Thé‡, Agnès Marchio‡,
Pierre Tiollais‡ & Anne Dejean‡

* Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cédex, France

‡ Unité de Recombinaison et Expression Génétique, Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris Cédex 15, France

We have previously described a human complementary DNA that encodes a novel protein which is homologous to members of the steroid/thyroid nuclear receptor multigene family¹. This novel protein (*hap* for hepatoma) exhibits strong homology with the human retinoic acid receptor (RAR)^{1,2} which has been recently characterized^{2,3}. To test the possibility that the *hap* protein might also be a retinoid receptor, a chimaeric receptor was created by replacing the putative DNA binding domain of *hap* with that of the human oestrogen receptor (ER). The resulting *hap*-ER chimaera was then tested for its ability to trans-activate an oestrogen-responsive reporter gene (vit-tk-CAT) in the presence of possible receptor ligands. Here we show that retinoic acid (RA) at physiological concentrations is effective in inducing the expression of this reporter gene by the *hap*-ER chimaeric receptor. This demonstrates the existence of two human retinoic acid receptors designated RAR- α and RAR- β .

† To whom correspondence should be addressed.

Fig. 1 Schematic representation of the homology between RAR- α and RAR- β and the structures of RAR-ER chimaeric receptors. In agreement with the report of Giguère *et al.*³, RAR- α (b) is represented here as a 462 amino-acid long protein (that is, 30 amino acids longer at the N-terminus than in our initial report². We have found (unpublished results) that the sequences of the RAR- α cDNA clones in our previous report² were not colinear with the corresponding genomic sequence upstream of our initiating AUG; in contrast perfect colinearity exists between the 5' terminal region of the cDNA sequence of Giguère *et al.*³ and our genomic sequence, substantiating their characterization of the open reading frame (ORF) of RAR- α cDNA). The receptors RAR- α (b) and RAR- β (c) are divided into six regions, A-F (a), by analogy with oestrogen receptors (see text). Numbers denote amino-acid positions. The circled numbers mark the positions of one exon junction, determined from genomic DNA sequence for RAR- α (ref. 2 and unpublished data of the Strasbourg laboratory) and RAR- β ¹⁰. Region E comprises the putative RA binding domain for each receptor. The degree of homology between the receptors is shown between (b) and (c) (% amino-acid identity). d The oestrogen receptor DNA-binding cassette (ER.CAS) of the hER construct HE28⁶ comprises the hER C-region (residues 185–205), flanked by unique restriction enzyme sites for *Kpn*I (5') and *Xho*I (3'). The corresponding sites were engineered on either side of region C of both a truncated form of RAR- α ² and RAR- β , allowing replacement of those regions by ER.CAS, creating the chimaeric receptors RAR- α -ER.CAS² (e) and RAR- β -ER.CAS (f).

Methods. The construction of RAR- α -ER.CAS (e) has been described². RAR- β -ER.CAS was assembled as follows. A 1.4 kilobase DNA fragment containing the entire RAR- β ORF was isolated from a partial digest of the clone λ 13 (ref. 1) with *Mae*I; the protruding ends were filled in with Klenow polymerase and the fragment was ligated initially into the *Sma*I site of pTZ18U (United States Biochemicals), yielding the plasmid pCOD20¹. For mutagenesis and expression studies, the insert was excized from pCOD20 by total digestion with *Bam*HI and partial digestion with *Eco*RI, and re-inserted into the *Eco*RI and *Bam*HI sites of the expression vector pSG5¹¹ yielding RAR- β 0, which can be used to express RAR- β *in vivo* and *in vitro*. Using oligonucleotide-directed mutagenesis as described for RAR- α ², *Kpn*I and *Xho*I sites flanking region C (codons 81–146) were created in RAR- β 0 whereas the *Xho*I site present in the A/B region was removed by mutation. RAR- β region C was then excized and replaced by the ER.CAS, giving the chimaeric receptor RAR- β -ER.CAS (f).

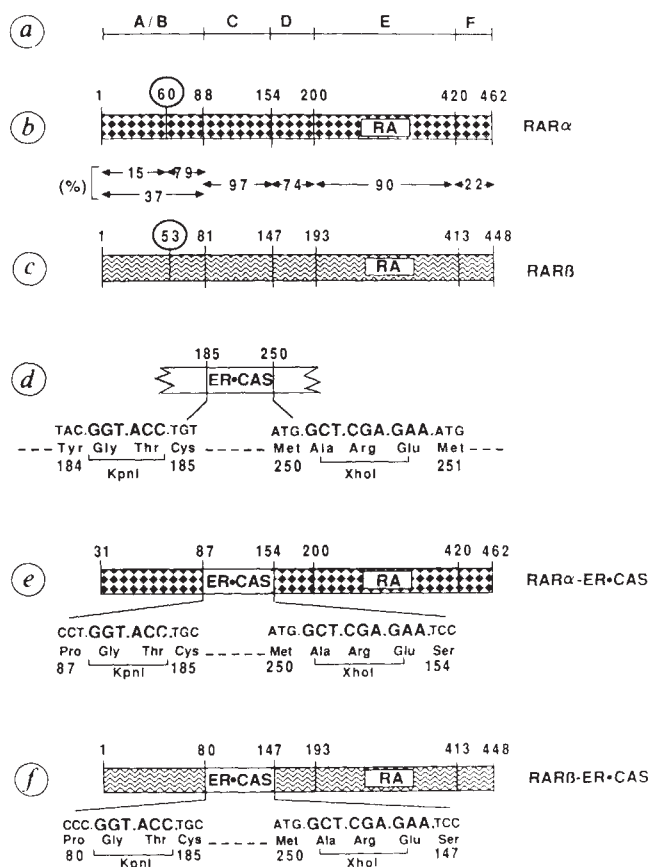


Figure 1, a-c shows a schematic comparison of the cDNA-deduced amino-acid sequences of RAR^{2,3} and *hap*¹ proteins (hereafter referred to as RAR- α and RAR- β , respectively). Note that RAR- α is represented as the full-length protein of 462 amino-acid residues³, instead of the form that we described previously, which lacks 30 amino acids at the N-terminus (ref. 2, see also legend to Fig. 1). RAR- α and RAR- β can be divided into five regions analogous to the A/B, C, D, E and F regions of other members of the nuclear receptor family (refs 4 and 5, Fig. 1a). The highest degree of homology (97% amino-acid identity) is found within the 66 residue region C, which in the case of the human glucocorticoid (hGR) and oestrogen (hER) receptors was identified as the DNA-binding domain responsible for the specific recognition of the cognate hormone-responsive elements (refs 6–9 and refs therein). The next most highly conserved region, E (90% amino-acid identity), is a stretch of 220 amino acids which is homologous to the ligand binding domain of the steroid hormone receptors and appears to contain the RA binding domain of RAR- α (ref. 2 and refs therein). Regions C and E are linked by a 46-residue hydrophilic region, D, which is 74% homologous between RAR- α and RAR- β . In contrast, the carboxy-terminal (F) and amino-terminal (A/B) portions of the receptors are much less similar (~22% and 37% amino-acid identity, respectively) and differ in length. A closer comparison of the A/B regions shows that residues 60–87 of RAR- α and 53–80 of RAR- β are 79% identical, whereas there is no significant homology within the remainder of the A/B region. Note in this respect that genomic DNA sequence analyses have located exon boundaries between residues 59/60 of RAR- α ² and residues 52/53 of RAR- β ¹⁰.

These structural homologies suggested to us that, like RAR- α , RAR- β might be a retinoid-inducible transcription factor. To test this hypothesis, we constructed a chimaeric receptor between RAR- β and the human oestrogen receptor, RAR- β -ER.CAS (Fig. 1f) in a similar experiment to that used to demonstrate that RAR- α encodes a receptor for RA² (see legend to Fig. 1 and ref. 11). To test the efficacy of various ligands to activate the chimaeric receptor, HeLa cells were co-transfected with RAR- β -ER.CAS and a reporter gene containing the oestrogen-responsive upstream sequence of *Xenopus* vitellogenin A2 gene ERE (*vit*) linked to the herpes simplex virus thymidine kinase (*tk*) promoter and the *Escherichia coli* chloramphenicol acetyltransferase gene (CAT) (*vit-tk-CAT*⁶). Addition to the culture medium of 10⁻⁷M of either thyroid hormone (T3 or T4), vitamin D3 [1,25(OH)₂D3], testosterone or oestradiol did not result in any stimulation of *vit-tk-CAT* expression by RAR- β -ER.CAS (data not shown). In contrast, a strong stimulation of CAT activity was observed in the presence of 10⁻⁷M RA, whereas a much weaker stimulation was achieved with the same concentration of retinol (Fig. 2 and data not shown). The extent of stimulation increased with increasing amounts of transfected RAR- β -ER.CAS with a maximum stimulation of at least 50-fold (Fig. 2a and c, left panel). This stimulation was similar to that obtained in transfection experiments where the previously described RAR- α chimaeric receptor, RAR- α -ER.CAS, was used to stimulate *vit-tk-CAT* expression². No increase in CAT activity was observed when the RAR- β expression vector RAR- β 0 was co-transfected with the *vit-tk-CAT* reporter gene instead of RAR- β -ER.CAS (data not shown).

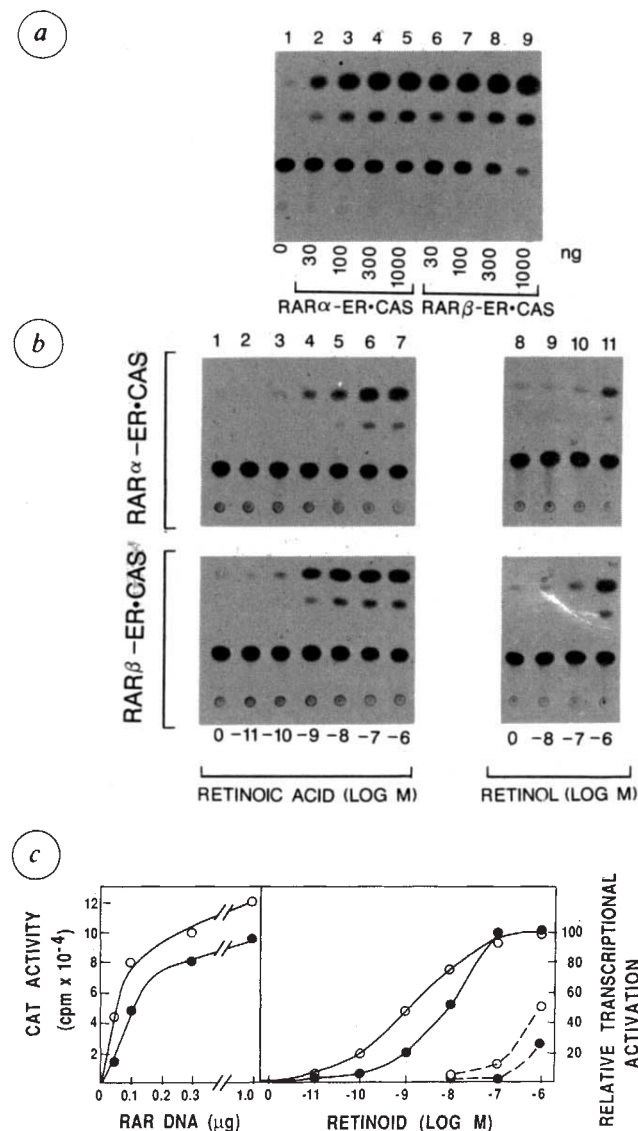


Fig. 2 *a*, CAT activity resulting from activation of the reporter gene *vit-tk-CAT* by the chimaeric receptors RAR-α-ER.CAS and RAR-β-ER.CAS in the presence of RA. From 0–1,000 ng of RAR-α-ER.CAS or RAR-β-ER.CAS, together with 2 μg of *vit-tk-CAT*, were transfected into HeLa cells which were subsequently treated with 10⁻⁷M RA. *b*, Effect of RA concentration on the induction of CAT activity by either RAR-α-ER.CAS or RAR-β-ER.CAS. HeLa cells were transfected with 30 ng of either RAR-α-ER.CAS or RAR-β-ER.CAS along with 2 μg of both *vit-tk-CAT* and β-galactosidase control plasmid pCH110 (see below) and then treated with the indicated concentrations of RA or retinol. *c*, **Left panel:** trans-activation of *vit-tk-CAT* by increasing concentrations of RAR-α-ER.CAS (●) or RAR-β-ER.CAS (○). Experiments were similar to those described under (*a*), except that the acetylated forms of ¹⁴C-chloramphenicol were isolated from the thin-layer chromatography plates and their radioactivities determined by scintillation counting. **Right panel:** Graph of the results shown in (*b*) for RAR-α-ER.CAS (●) and RAR-β-ER.CAS (○) in response to RA (—) or retinol (---). Experiments were performed as in (*b*), except that the acetylated forms of ¹⁴C-chloramphenicol were isolated, quantified by scintillation counting and the results expressed in per cent of maximal activation. The results displayed in both left and right panels are representative of several independent transfection experiments which gave identical results within 10% variation.

Methods. Transfection experiments were as described². In (*a*) 2 μg of *vit-tk-CAT* reporter DNA, 2 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) and the indicated amounts of the RAR-α or RAR-β chimaeric DNA (plus 16 μg of carrier plasmid DNA) were transfected into HeLa cells. In (*b*) transfections were as in (*a*), but with 30 ng of RAR-α- or RAR-β-ER.CAS. Aliquots of extracts prepared from the transfected cells and corresponding to 1 OD unit of β-galactosidase activity were assayed for CAT activity as previously described².

To demonstrate directly that RAR-β binds RA, cytoplasmic extracts prepared from COS-1 cells transfected with the RAR-β0 expression vector were incubated with labelled RA in the presence or absence of excess unlabelled RA or retinol, as described for RAR-α². An increase in the specific, high affinity binding of RA was observed in the transfected cell extracts. As for RAR-α², however, there was high background binding in extracts of untransfected cells due to endogenous cellular retinoic acid binding protein (CRABP), thus precluding any accurate determination of the affinity of RA for its receptors (data not shown). The relative affinity of RA for RAR-α and RAR-β was therefore estimated by measuring the activation of *vit-tk-CAT* expression as a function of the ligand concentration under conditions where the reporter gene was present in large excess over the chimaeric receptor (30 ng of RAR-α-ER.CAS or RAR-β-ER.CAS per transfection, see Fig. 2*a* and *c*, left panel). Under these conditions, the concentration of ligand leading to 50% of the maximum inducible CAT activity (ED₅₀) should reflect the relative affinity of the two chimaeric receptors for RA. In agreement with our previous report², the ED₅₀ for the RAR-α chimaera was close to 10⁻⁸M (Fig. 2*b* and *c*, right panel). In contrast, efficiency of RA in stimulating CAT activity was consistently ~10-fold greater with the RAR-β chimaera (ED₅₀ ~10⁻⁹M), suggesting that RAR-β has a 10-fold greater affinity for RA than RAR-α does. Note that for both RAR-α

and RAR-β chimaeras, retinol was ~1,000-fold less efficient than RA at stimulating expression of the reporter gene. As previously discussed for RAR-α, however, we cannot conclude that retinol is directly able to induce trans-activation by RAR-β, as it is known that retinol can be converted to RA in cultured cells¹².

The present data, together with our previous studies^{1,2} clearly establish the existence of two structurally closely-related human retinoic acid receptors encoded in two distinct genes which map to different chromosomes. The gene encoding the previously characterized retinoic acid receptor^{2,3}, designated here as RAR-α, maps to chromosome 17q21.1 (refs 2, 13), whereas the receptor RAR-β, formerly called *hap*, is encoded in a gene that maps to chromosome 3p24 (Mattei, M. G., H. d.T., A.M., P.T. and A.D., manuscript submitted). It is interesting that RAR-α and RAR-β are more homologous to the two closely-related thyroid hormone receptors TRα and TRβ, located on chromosomes 17q11.2 (refs 2, 13) and 3p21–25 (refs 14, 15) respectively, than to any other members of the nuclear receptor family (refs 1–3). These observations suggest that the thyroid hormone and retinoic acid receptors have evolved by gene, and possibly chromosome, duplications from a common ancestor which itself diverged rather early in evolution from the common ancestor of the steroid receptor group of the family. In this respect, we note that the counterparts of the human RAR-α and RAR-β

genes are present in both mouse and chicken genomes (unpublished results).

In view of the multiple effects of retinoids on both animal development and homeostasis^{2,3,16,17}, the identification of a second retinoic acid receptor raises a number of interesting questions. In what tissues are these receptors expressed, to what levels and at what point in development? Preliminary Northern blot analyses of mouse and chicken RNAs from various tissues indicate some specificity in their distribution (unpublished results). Obviously, important clues to the mechanisms through which retinoids control many developmental and homeostatic processes will be obtained by determining the spatial and temporal patterns of expression of the various elements of the retinoid signal transduction system, including both the α and β receptors and the cellular retinoic acid and retinol binding proteins (CRABP and CRBP^{2,3,17}). Do RAR- α and RAR- β trans-activate the transcription of different sets of target genes? The high degree of homology between their putative DNA-binding domains (region C, 97% amino-acid identity) suggests that the two receptors might recognize a common RA-responsive element. Their difference in the A/B region, however, may result in differential gene activation, as the corresponding region of the human oestrogen receptor appears to play a specific role in the activation of different oestrogen-responsive genes⁷. Unfortunately, no promoter regions of genes transcriptionally controlled by RA are presently available to test these possibilities.

Our results indicate that RAR- β may mediate activation of transcription by RA at concentrations 10-fold lower than those necessary for activation by RAR- α , although both receptors respond to RA concentrations within the range observed for RA action *in vivo*. As the ED₅₀ values for the various biological effects of RA in cell culture span a wide range of concentrations (4×10^{-10} to $>10^{-8}$ M (ref. 18)), it is possible that the two RA receptors may be differentially involved in these effects. A concentration gradient of RA across the anterior-posterior axis of the developing chick-embryo limb-bud has recently been described¹⁹, implicating RA as the morphogen in this system. The existence of two receptors for RA, possibly differing in their affinities for the ligand, might play a role in the interpretation of morphogenetic gradients.

We thank Dr S. Green for pSG5 and ER.CAS, Hoffmann-La Roche for vitamin D3, A. Staub and F. Ruffenach for oligonucleotides, the tissue culture lab for cells, C. Werlé and B. Boulay for figures and the secretariat for typing the manuscript. This research was supported in Strasbourg for the INSERM, the CNRS, the Ministère de la Recherche et de l'Enseignement Supérieur, the Fondation pour la Recherche Médicale and the Association pour la Recherche sur le Cancer, and in Paris by grants from the NIH, the Ligue Nationale Française contre le Cancer, the FRM and the ARC. M.P. has a fellowship from the MRC of Canada, and N.B. one from EMBO.

Received 26 February; accepted 21 March 1988.

- de Thé, H., Marchio, A., Tiollais, P. & Dejean, A. *Nature* **330**, 667-670 (1987).
- Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. *Nature* **330**, 444-450 (1987).
- Giguere, V., Ong, E. S., Segui, P. & Evans, R. M. *Nature* **330**, 624-629 (1987).
- Krust, A. *et al.* *EMBO J.* **5**, 891-897 (1986).
- Green, S. & Chambon, P. *Nature* **324**, 615-617 (1986).
- Green, S. & Chambon, P. *Nature* **325**, 75-78 (1987).
- Kumar, V. *et al.* *Cell* **51**, 941-951 (1987).
- Hollenberg, S. M., Giguere, V., Segui, P. & Evans, R. M. *Cell* **49**, 39-46 (1987).
- Rusconi, S. & Yamamoto, K. R. *EMBO J.* **6**, 1309-1315 (1987).
- Dejean, A., Bouguier, L., Grzeschik, K. H. & Tiollais, P. *Nature* **322**, 70-72 (1986).
- Green, S., Issemann, I. & Scheer, E. *Nucleic Acids Res.* **16**, 369 (1988).
- Williams, J. B. & Napoli, J. L. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4658-4662 (1985).
- Mattei, M. G., Petkovich, M., Mattei, J. F., Brand, N. & Chambon, P. *Hum. Genet.* (in the press).
- Thompson, C. C., Weinberger, C., Lebo, R. & Evans, R. M. *Science* **237**, 1610-1614 (1987).
- Garcia, J. L., Houle, B., Ledue, F., Bradley, W. E. C. & Dobrovic, A. *Nucleic Acids Res.* **16**, 1223 (1988).
- Robertson, M. *Nature* **330**, 420-421 (1987).
- Sporn, M. B., Roberts, A. B. & Goodman, D. S. (eds) *The Retinoids* (Academic, Florida, 1984).
- Sporn, M. B. & Roberts, A. B. in *The Retinoids* Vol. 1 (eds Sporn, M. B., Roberts, A. B. & Goodman, D. S.) 235-279 (Academic, Florida, 1984).
- Thaller, C. & Eichele, G. *Nature* **327**, 625-628 (1987).

GAL4 activates transcription in *Drosophila*

Janice A. Fischer*, Edward Giniger*, Tom Maniatis & Mark Ptashne

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA

GAL4 is a yeast regulatory protein that binds to specific sites within a DNA sequence called UAS_G (galactose upstream activating sequence) and activates transcription of linked genes¹⁻⁶. This activation requires two functions of the protein^{7,8}: a DNA binding domain located near the amino terminus⁸, and one or more 'activating regions'⁷⁻¹¹. The 'activating regions' are highly acidic⁹⁻¹¹ (see also ref. 12) and can be replaced, for example, by a short peptide designed to form a negatively charged, amphipathic α -helix¹³. GAL4, as well as deletion derivatives bearing one or more 'activating regions' attached to the DNA binding domain, activates transcription in cultured mammalian cells from mammalian promoters linked to a UAS_G (refs 14, 15). Here we show that GAL4, when expressed in particular tissues of *Drosophila* larvae, stimulates tissue-specific transcription of a *Drosophila* promoter linked to GAL4 binding sites.

We constructed an effector gene that expresses GAL4 in a tissue specific manner in *Drosophila*, and a reporter gene that allows the visualization of GAL4-activated transcription by a histochemical stain for β -galactosidase activity (Fig. 1). In the effector gene, expression of GAL4 is driven by a *Drosophila* *Adh* promoter. This promoter, when fused to the *lacZ* gene of *Escherichia coli*, expresses β -galactosidase mainly in four larval tissues—fat body (fb), Malpighian tubules (mt), anterior midgut (amg) and middle midgut (mmg); expression in the hindgut (hg) and tracheae (tr) is variable (Figs 3, 4; refs 16, 34). In the reporter gene, the TATA-box and 5'-untranslated region of the *Drosophila* heat shock gene *hsp70* are fused to *lacZ* (Fig. 1). This fusion gene is transcriptionally inactive (see below): the *hsp70* promoter is normally heat inducible, but sequences upstream of -43 from the *hsp70* transcription start site were deleted, thus eliminating the regulatory elements required for that induction¹⁷⁻²⁰. We inserted four copies of a 17 base pair (bp) sequence called the 17-mer upstream of the *hsp70* TATA-box: these 17-mers are closely related to the GAL4 binding sites in UAS_G (ref. 8) and are recognized by GAL4 (ref. 15) (see legend to Fig. 1).

The effector and reporter genes were separately introduced into the *D. melanogaster* genome by P element transformation^{21,22} (Fig. 1 legend). Three independent effector transformant lines were separately mated to nine independent reporter transformant lines. Twelve larval progeny of each cross were tested for expression of the reporter gene by a histochemical staining assay for β -galactosidase activity (Fig. 4 legend). The flies transformed with the effector and reporter genes were each heterozygous for the respective P elements (Fig. 1 legend), and so we expected one fourth of their larval progeny to carry one copy of each gene.

In each of the 27 crosses, we detected β -galactosidase activity in ~one quarter of the larval progeny. In all of these larvae, enzyme activity was observed at high levels in the fat body and anterior midgut (Fig. 2), two of the tissues in which we expect the *Adh* promoter of the effector gene to be active. Also as expected, we detected variable levels of β -galactosidase activity in the hindgut and tracheae (Figs 2 and 3). In the middle midgut

* Present addresses: Howard Hughes Medical Institute, Department of Biochemistry, University of California, Berkeley, California 94720, USA (J.A.F.); Howard Hughes Medical Institute, Department of Physiology, University of California, San Francisco, California 94143, USA (E.G.).

Fig. 1 Effector and reporter genes introduced into the *D. melanogaster* genome by P element transformation. The effector gene (*Adh/GAL4*) is a hybrid gene in which a *Drosophila Adh* promoter drives transcription of the yeast GAL4 coding sequences. The *Adh* promoter in the effector gene is that of the *D. mulleri Adh-1* gene^{24,25}, with additional copies of its natural enhancer, BOX B^{16,34} inserted upstream to increase its level of expression. The reporter gene (*17-hsp70/lacZ*) is a hybrid gene in which the TATA-box of the *Drosophila hsp70* gene, with four GAL4 17-mers immediately upstream, drives transcription of the *E. coli* gene encoding β -galactosidase (*lacZ*). The 17-mers are high affinity GAL4-binding sites (see below). Each gene contains transcriptional termination sequences from *hsp70*. The effector and reporter genes were separately introduced into the *D. melanogaster* genome by P element transformation^{21,22}.

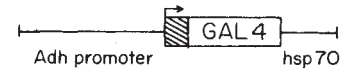
Methods. The *Adh* promoter in the effector gene contains a *Bgl*II/*Bal*I fragment from the 5'-flanking region of the *D. mulleri Adh-1* gene which extends from -1.5 kilobase (kb) to +58 bp from the transcription start site, immediately downstream of the ATG start codon. A *Bam*HI linker was inserted at the *Bal*I site, and three copies of an 100 bp *Sac*I-linked DNA fragment (-282 to -181 from the *Adh-1* transcription start site) containing BOX B were cloned into the *Sna*BI site at -1.45 kb. GAL4 coding sequences were fused to the *Adh* promoter at the *Bam*HI site inserted downstream of the ATG of *Adh-1* via a *Hind*III site within the untranslated leader sequence of GAL4 in pLPK-C15²⁶. Site-directed mutagenesis with a mismatched primer²⁷ generated a 'perfect' fusion of the *Adh-1* leader sequences to the ATG start codon of GAL4. The reporter gene was constructed from an *hsp70/lacZ* gene (a gift of John Lis and colleagues) containing *Drosophila hsp70* sequences from -43, 10 bp upstream of the TATA-box, to +265 (thus including the promoter and the first seven codons), fused in-frame to an *E. coli lacZ* gene. Four copies of a 29 bp *Kpn*I/*Pst*I fragment of pMH100¹⁵, containing a high affinity (Melvyn Hollis and M. Ptashne., unpublished data; see ref. 15) 17-mer GAL4 recognition site (the *Sca*I 17-mer: 5'-CGGAGTACTGTCTCCG-3') were cloned into the *Kpn*I site just upstream of the *hsp70/lacZ* gene in pSP73lac2 (a gift of Dean Falb), which contains the *hsp70/lacZ* gene cloned as a *Sal*I fragment into the *Sal*I site of pSP73 (ref. 28). A 4.8 kb *Xba*I fragment containing the effector gene and a 3.6 kb *Xba*I fragment containing the reporter gene were each cloned into the P element transformation vector C70TX (a gift of Dean Falb), in the same transcriptional orientation as the *rosy* gene. C70TX was constructed by inserting an *Xba*I linker into the *Sal*I site of C70T (a gift of John Lis and colleagues), which is a derivative of Carnegie 20 (ref. 29) containing, in the *Sal*I site, a 250 bp *Sal*I/*Xho*I fragment of *hsp70* gene terminator sequences. The P element plasmids were purified by banding in CsCl-EtBr gradients and coinjected at a concentration of 300 μ g ml⁻¹ with the helper plasmid p π 25.7wc³⁰ (70 μ g ml⁻¹) into embryos of *D. melanogaster* strain *ry*⁵⁰⁶ as described^{21,22}. The effector plasmid was also injected at a concentration of 100 μ g ml⁻¹, because very few embryos injected even with the lower concentration survived past the first instar larval stage. Flies from embryos that survived microinjection were individually backcrossed to *ry*⁵⁰⁶. Among the backcross progeny, germ-line transformants were distinguished by their wild type (*ry*⁺) eye colour. The backcross progeny, heterozygous for the P element, were used to perform the effector $\times$ reporter crosses. All enzymatic reactions and DNA manipulations were carried out using standard conditions and techniques³¹. Flies were grown at 25 °C on standard cornmeal food.

Fig. 2 Reporter gene transcripts in transformed larvae. The transcription start site of the reporter gene (*17-hsp70/lacZ*) in the transformed larvae or larval progeny of the crosses indicated was assayed by quantitative RNase protection³² using the uniformly ³²P-labelled RNA probe shown, complementary to the 5' end of the reporter transcripts, and also an RNA probe complementary to the endogenous α 1-tubulin gene as an internal control for mRNA levels. ENH-*hsp70/lacZ* (lane 1) are transformants (a gift of Dean Falb) carrying a gene like the reporter, except the enhancer of the *D. melanogaster Adh* gene's distal promoter (-660 to -128 from the distal transcription start site; Dean Falb and T.M., unpublished data) was installed upstream of the truncated *hsp70* promoter instead of the GAL4 17-mers. Two independent effector (*Adh/GAL4*) transformant lines were crossed with the same reporter (*17-hsp70/lacZ*) line in lanes 2 and 3. Identical fragments of the reporter transcripts were protected by the probe in lanes 1-3. The appearance of two bands is probably an artefact of the RNase digestion. Consistent with the β -galactosidase activity assay, no protected fragments were detected in larval progeny of a cross of the effector and a reporter with no 17-mers (*hsp70/lacZ*; lane 4), or in larvae transformed with the reporter gene alone (lane 5). (Flies transformed with the *hsp70/lacZ* gene, which lacks the 17-mers, were a gift of Dean Falb.) An approximation of the relative strengths of the GAL4 enhancer and the particular *Drosophila Adh* enhancer in ENH-*hsp70/lacZ* was obtained by densitometry of appropriate autoradiographic exposures of the *hsp70/lacZ* and tubulin protected fragments in lanes 1-3. The ratios of *hsp70/lacZ* to tubulin signals were corrected for gene copy number and then normalized to the ratio obtained for lane 1, which was arbitrarily assigned the value 1.0: the results were 0.3 (lane 2) and 1.2 (lane 3). Thus, GAL4 bound to the 17-mers is comparable with the *Adh* enhancer in its ability to activate transcription of the reporter gene.

Methods. RNA was prepared as previously described^{24,25} from actively feeding third instar larvae, or from ENH-*hsp70/lacZ* adults. Two uniformly ³²P-labelled RNA probes were transcribed with SP6 RNA polymerase as described³³, from the plasmids SP6- α tub²⁵ (a gift of Vicki Corbin) and SP6-hslac. SP6-hslac was constructed by ligating a ~450 bp *Bgl*II/*Bgl*I fragment (the *Bgl*II end was treated with T4 DNA polymerase) of pSP73lac2 (see Fig. 1 legend) into pSP72 (ref. 28) digested with *Bgl*II and *Sma*I. Before transcription, SP6-hslac was digested with *Bgl*II, resulting in a ~450 nucleotide (nt) probe. The SP6- α tub probe²⁵ protected a 90 nt fragment of the endogenous α 1-tubulin gene transcripts. As shown, the SP6-hslac probe protected a 408 nt fragment of the *hsp70/lacZ* fusion gene transcripts. Five-forty μ g of each RNA preparation was hybridized simultaneously with the ³²P-labelled RNA probes as previously described²⁵. RNase digestion conditions were also as described²⁵. Samples were electrophoresed on a 6% denaturing acrylamide gel. The top portion of the gel is a longer autoradiographic exposure than the bottom portion.

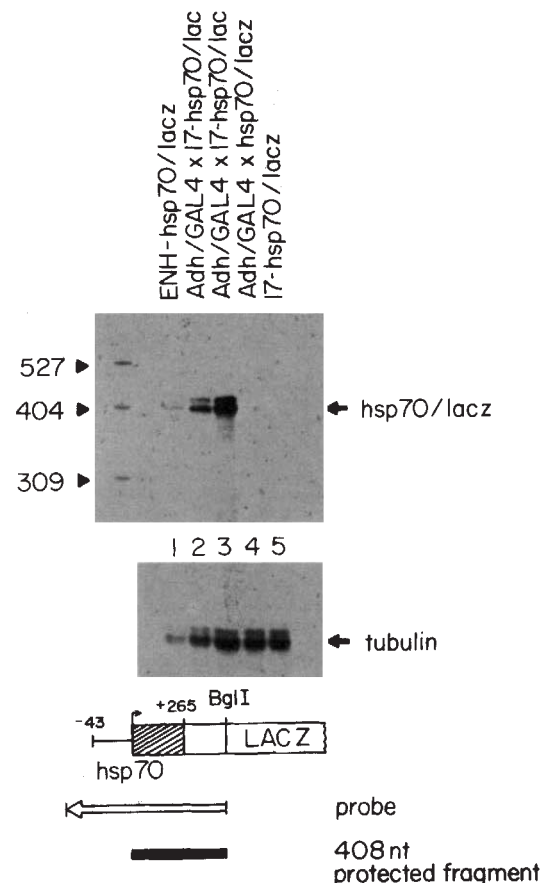
Effector

Adh / GAL4



Reporter

17-hsp70 / lacZ



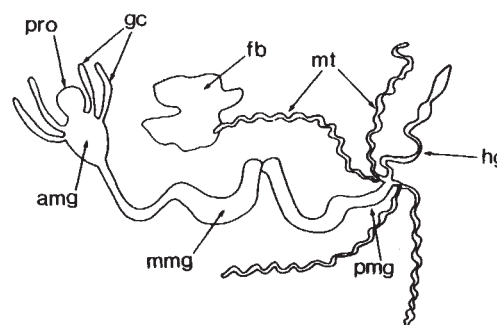
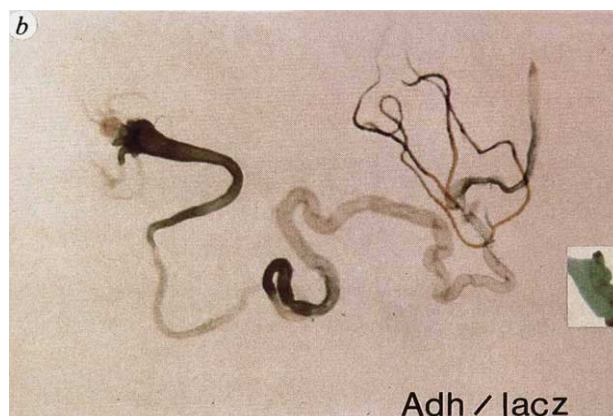
Summary of β -galactosidase Activity
in Larval Tissues

	fb	mt	amg	mmg	hg	tr	gc	pro	pmg	di	br	sg	ov/ts
<i>Adh/lacZ</i> (6)	+	+	+	+	var	var	-	-	-	-	-	-	-
<i>Adh/GAL4</i> (3); 17- <i>hsp70/lacZ</i> (9)	+	+/-	+	+/-	var	var	-	-	-	-	-	-	-

Fig. 3 Tissue specificity of β -galactosidase activity in transformed larvae. The tissue specificity of β -galactosidase activity, detected by histochemical staining, in *Adh/lacZ* transformant larvae or in larvae carrying both the effector (*Adh/GAL4*) and reporter (*17-hsp70/lacZ*) genes is summarized. The *Adh/lacZ* gene is described in the legend to Fig. 4, and the effector and reporter genes are described in Fig. 1. The numbers in parentheses indicate the number of independent transformant lines assayed. The tissues indicated are: fat body (fb), Malpighian tubules (mt), anterior midgut (amg), middle midgut (mmg), hindgut (hg), tracheae (tr), gastric caecae (gc), proventriculus (pro), posterior midgut (pmg), imaginal discs (di), brain (br), salivary glands (sg), ovaries and testes (ov/ts). +, High staining intensities in all lines. -, Staining was never seen, +/-, Staining was observed in some larvae, and only at a low level in a few cells. Variable staining is indicated as var: by variable we mean that the staining intensity varied from high to none within or between different lines or crosses. No β -galactosidase activity was detected with a reporter gene lacking 17-mers, nor in larvae transformed with the reporter gene alone (data not shown).

Fig. 4 β -galactosidase activity in larval tissues of *P. element* transformants. *a*, Diagram of the larval tissues in the photographs beneath: gastric caecae (gc), proventriculus (pro), anterior midgut (amg), middle midgut (mmg), posterior midgut (pmg), Malpighian tubules (mt), fat body (fb), and hindgut (hg) are indicated. *b*, β -galactosidase activity in third instar larvae of six independent lines transformed with an *Adh/lacZ* hybrid gene was visualized by a histochemical stain. The hybrid gene contains the same *Adh* promoter as in the effector gene, but here, fused in-frame to the *E. coli lacZ* gene rather than to *GAL4*^{16,34}. Blue staining is observed only in the fat body, Malpighian tubules, anterior midgut, middle midgut, and in this particular larva, also in the hindgut. Expression of β -galactosidase in the hindgut, and also in the tracheae, was variable (see refs 16, 34 and Fig. 3). *c*, A third instar larva carrying both the effector (*Adh/GAL4*) and the reporter (*17-hsp70/lacZ*) genes expressed β -galactosidase in the fat body and anterior midgut. Other larvae expressed β -galactosidase in the hindgut and/or tracheae, and low levels of β -galactosidase activity were sometimes detected in a few cells of the middle midgut or Malpighian tubules (see Fig. 3).

Methods. Transformant larvae were dissected in a solution of 1% glutaraldehyde in 0.1 M NaPO₄ pH 7.0, 1 mM MgCl₂. After 15 min, the tissues were transferred to a stain solution consisting of 10 mM NaPO₄ pH 7.0, 150 mM NaCl, 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆ 3H₂O, 3.3 mM K₄Fe(CN)₆ 3H₂O, and 0.2% X-gal dissolved as a 2% solution in dimethylformamide (Pieter Wensink, personal communication). After staining for 15 min (*a*) or 1 h (*b*), the tissues were placed in a drop of 70% glycerol on a slide, covered with a coverslip and photographed.

a*b**c*

and Malpighian tubules, two other tissues in which we expect the *Adh* promoter of the effector gene to be active, we detected enzyme activity in only a small number of larvae, and then only in a few cells (data not shown); perhaps the *Adh/GAL4* transcript or *GAL4* itself is particularly unstable in these two tissues. β -galactosidase activity was never detected in tissues other than those in which we expect the *Adh* promoter of the effector gene to be active (Figs 3 and 4), and no β -galactosidase activity (data not shown) nor reporter transcripts (Fig. 2) were detected in larvae transformed with the reporter gene alone. Furthermore, neither β -galactosidase activity (data not shown; see Fig. 3) nor transcripts (Fig. 2) were detected from a reporter gene lacking the 17-mers. *GAL4*-activated transcription of the reporter gene was initiated at the same start point as was transcription of the reporter gene activated by a *Drosophila Adh* enhancer (Fig. 2).

GAL4 bound to the 17-mers stimulated transcription as efficiently as the *Drosophila* enhancer (Fig. 2 legend).

We have shown that a single protein, *GAL4*, when expressed in a tissue specific manner, generates tissue specific gene expression in *Drosophila*. Typical tissue specific enhancers (the best characterized are those of mammalian genes) interact with several different proteins (ref. 23 and refs therein). Our results suggest that the apparent complexity of tissue specific enhancers is not necessary for their function as transcriptional activators. Perhaps that complexity is exploited to obtain intricate patterns of gene control from a relatively small number of regulatory proteins. The ability of *GAL4* to activate mammalian and *Drosophila* promoters suggests that the protein with which DNA-bound *GAL4* interacts to stimulate transcription in yeast—perhaps RNA polymerase—has a close homologue in

higher eukaryotes. It seems likely that the mechanism of action of at least one class of gene activators is conserved between yeast, mammalian cells and *Drosophila*.

We thank Dean Falb for generous gifts of unpublished plasmids and transformant lines, and Gerald Rubin, Melvyn Hollis, Liam Keegan and John Lis for plasmids. This work was supported by grants from the American Cancer Society (to M.P.) and the National Institutes of Health (to T.M.).

Received 29 February; accepted 14 March 1988.

- Guarente, L., Yocum, R. R. & Gifford, P. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7410-7414 (1982).
- Yocum, R. R., Hanley, S., West, R. W., Jr. & Ptashne, M. *Molec. cell. Biol.* **4**, 1985-1988 (1984).
- West, R. W., Jr., Yocum, R. & Ptashne, M. *Molec. cell. Biol.* **4**, 2467-2478 (1984).
- Johnston, M. & Davis, R. W. *Molec. cell. Biol.* **4**, 1440-1448 (1984).
- Bram, R. & Kornberg, R. D. *Proc. natn. Acad. Sci. U.S.A.* **82**, 43-47 (1985).
- Giniger, E., Varnum, S. M. & Ptashne, M. *Cell* **40**, 767-774 (1985).
- Brent, R. & Ptashne, M. *Cell* **43**, 729-736 (1985).
- Keegan, L., Gill, G. & Ptashne, M. *Science* **231**, 699-704 (1986).
- Ma, J. & Ptashne, M. *Cell* **48**, 847-853 (1987).
- Ma, J. & Ptashne, M. *Cell* **51**, 113-119 (1987).
- Gill, G. & Ptashne, M. *Cell* **51**, 121-126 (1987).
- Hope, I. A. & Struhl, K. *Cell* **46**, 885-894 (1986).
- Giniger, E. & Ptashne, M. *Nature* **330**, 670-672 (1987).
- Kakidani, H. & Ptashne, M. *Cell* **52**, 161-167 (1987).
- Webster, N., Jin, J. R., Green, S., Hollis, M. & Chambon, P. *Cell* **52**, 169-178 (1987).
- Fischer, J. A. thesis Harvard Univ. (1987).
- Pelham, H. R. B. *Cell* **30**, 517-538 (1982).
- Lis, J. T., Simon, J. A. & Sutton, C. A. *Cell* **35**, 403-410 (1983).
- Dudler, R. & Travers, A. A. *Cell* **38**, 391-398 (1984).
- Cohen, R. S. & Meselson, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5509-5513 (1984).
- Spradling, A. C. & Rubin, G. M. *Science* **218**, 341-347 (1982).
- Rubin, G. M. & Spradling, A. C. *Science* **218**, 348-353 (1982).
- Maniatis, T., Goodbourn, S. & Fischer, J. A. *Science* **236**, 1237-1245 (1987).
- Fischer, J. A. & Maniatis, T. *Nucleic Acids Res.* **13**, 6899-6917 (1985).
- Fischer, J. A. & Maniatis, T. *EMBO J.* **5**, 1275-1289 (1986).
- Silver, P. A., Keegan, L. P. & Ptashne, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5951-5955 (1984).
- Zoller, M. J. & Smith, M. *Meth. Enzym.* **100**, 469-500 (1983).
- Krieg, P. A. & Melton, D. A. *Meth. Enzym. Recomb. DNA* **155**, 397-415 (1987).
- Rubin, G. M. & Spradling, A. C. *Nucleic Acids Res.* **11**, 6341-6351 (1983).
- Karess, R. E. & Rubin, G. M. *Cell* **38**, 135-146 (1984).
- Maniatis, T., Fritsch, E. F., Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
- Zinn, K., DiMaio, P. & Maniatis, T. *Cell* **34**, 865-879 (1983).
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. *Nucleic Acids Res.* **12**, 7035-7056.
- Fischer, J. A. & Maniatis, T. *Cell* **53** (in the press).

Periodic interactions of heat shock transcriptional elements

Robert S. Cohen* & Matthew Meselson

Department of Biochemistry and Molecular Biology,
Harvard University, Cambridge, Massachusetts 02138, USA

The activity of some genes is known to be a periodic function of the amount of DNA between the binding sites of its regulatory proteins. The period observed is close to 10.5 base pairs (bp), that is, one turn of the B form of the DNA helix¹⁻³. Such periodicity is also seen in the cooperative binding of phage lambda and lac repressors^{4,5}. These periodic phenomena have been attributed to a requirement for a unique rotational alignment in forming essential contacts between the bound proteins^{1,6,7}. Here we report a strong periodic dependence of the transcription of a cloned *Drosophila melanogaster* heat shock gene on the amount of DNA inserted between its two heat shock consensus elements. In addition, we find a similar periodicity for insertions just 3' to the proximal heat shock element. Consistent with the torsional flexibility of DNA, these periodic effects are seen for short insertions, up to ~80 bp, but not for much longer ones. We conclude that maximal transcription requires rotationally unique contacts between proteins bound to the two heat shock elements and also requires correct alignment of additional sites of DNA-protein binding downstream of the proximal element.

Studies with transfected *Drosophila* tissue culture cells, trans-

genic flies and cell free systems indicate that maximal transcription of the *hsp70* heat shock gene requires two copies of a 14 bp heat shock element (HSE) with consensus sequence CnnGAAnnTTCnnG⁸⁻¹⁵. In response to heat shock, HSEs bind protein *in vivo*^{16,17} and a protein purified from *Drosophila* nuclei binds to HSEs and promotes the transcription of cloned heat shock genes *in vitro*^{18,19}. The purified protein, heat shock transcription factor (HSTF), binds successively and cooperatively to the two HSEs closest to the *hsp70* transcription initiation site, first to the more proximal or strong site and then to the adjacent weak site^{19,20}. The strong site (-62 to -49) matches the consensus HSE at all 8 positions while the weak site (-85 to -72) matches the consensus HSE at only 6. The apparent requirement for two HSEs and the cooperativity of HSTF binding led us to investigate the possibility that there is a requirement for specific rotational alignment of the two elements. Since HSTF and another protein together protect an additional 40-80 bp 3' to the proximal HSE^{16,17,21}, we also investigated the effect of rotating the region containing the two HSEs with respect to downstream sequences.

The transcription of a cloned *hsp70* gene in transfected heat-shocked *Drosophila* tissue culture cells was determined as a function of the spacing between the distal and proximal HSEs and also as a function of the spacing between the proximal HSE and sequences downstream. These two intervals were increased by inserting short DNA fragments that contributed either a near integral or non-integral multiple of 10.5 bp. The insertions either preserved or greatly altered the relative angular orientations of the adjacent sequences about the helical axis. In addition, the lengths of the two intervals were increased by inserting relatively long DNA fragments that differed from each other by fractions of a turn. The insertions between the HSEs (at the *Bss*II restriction site, Fig. 1) were 4, 10, 14, 20, 24, 26, 63, 77, 295, 296, 300, 302 and 304 bp. The insertions at the 3' end of the proximal HSE (at the *Nru*I restriction site) were 6, 10, 16, 20, 26, 30, 67, 73, 291, 292, 298, 300 and 302 bp.

As can be seen in the Northern blots from a representative experiment (Fig. 2a, b), and in the graph (Fig. 2c), which averages the data obtained from this and two other independent experiments, short insertions at the *Nru*I and *Bss*II site alter transcription in a periodic fashion. The length of the period is close to 10.5 bp, one helical turn of the B form of the helix. All of the short insertions that differ from an integral multiple of 10.5 by 1 bp or less (10, 20, 63 and 73 bp) reduced transcription by no more than a factor of two, whereas all insertions that differ from integral multiples by at least 3 bp (4, 6, 14, 16, 24, 26, 67 and 77 bp) reduced transcription 4- to 15-fold. In contrast to the periodic effect of small insertions, each of the ten large insertions (291-304 bp) reduced transcription to about 50% of the wild-type level, with no indication of periodicity.

The striking periodicity of ~10.5 bp that we see for short insertions indicates that maximal *hsp70* transcription requires specific rotational alignment of DNA-protein interaction sites flanking the *Bss*II and *Nru*I sites. The lack of periodicity for long insertions suggests that spacings of ~300 bp are sufficient to achieve correct rotational alignment by DNA twisting. This is consistent with the torsional flexibility of DNA determined from studies of supercoiling in physiological buffer^{22,23}. For example, a free energy of 2 kcal gives a calculated twist of 180° for a 300 bp segment but only a tenth of this for a 30 bp segment.

The periodic effects of insertions at the *Bss*II and *Nru*I sites could reflect a requirement for unique rotational alignment of three bindings regions, one upstream of the *Bss*II site, one downstream of the *Nru*I and one between the two restriction sites. Another possibility is that only the two outside binding regions require such alignment. If the latter were true, an *hsp70* gene with a half-integral insertion at the *Bss*II site and another half-integral insertion at the *Nru*I site should be transcribed with approximately wild-type efficiency. As shown in Fig. 3, two such constructs are transcribed only about 1/20th as efficiently

* Present address: Department of Biochemistry and Molecular Biophysics, Urology, Columbia University, New York, New York 10032, USA.

Fig. 1 Top, map of the parental *hsp70-cat* transfection vector (not to scale). The *hsp70-cat* gene consists of the *hsp70* 5' region -146 to +89 (*EcoRI-PstI*) linked to an 857 bp segment of the *E. coli* CAT gene (*PstI-SacI*) followed by the *hsp70* 3' region +2027 to +2411 (*SacI-XhoI*). The *hsp70-cat* gene is cloned into the *XhoI-EcoRI* sites of plasmid pAPX²⁴. The sequence of the *EcoRI-PstI* *hsp70* segment is identical to that previously reported⁹. The 1.4 kilobase (kb) *hsp70-cat* transcriptional unit is indicated by the arrow. Restriction sites: R, *EcoRI*; B, *Bss*II; N, *Nru*I; S, *Sal*I; X, *Xho*I. The transfection vector containing the *hsp70-λ* gene (not shown) is identical to the *hsp70-cat* vector except that the former contains the 1,051 bp *PstI-SacI* fragment of phage-λ in place of the 857 bp *PstI-SacI* CAT fragment. Bottom, A list of the DNA sequences inserted between the distal and proximal HSEs (*Bss*II insertions) and at the 3' end of the proximal HSE (*Nru*I insertions). *Bss*II and *Nru*I cleave the DNA immediately downstream of nucleotides -68 and -51, respectively. All insertions at the *Nru*I site have a G in the second position, restoring the 8 out of 8 match of the proximal HSE to the consensus element. Sequence abbreviations: a, b, c, * represent the 49, 63, 57 and 281 bp pBR322 *Alu*I fragments, respectively; *1, *2, *3 and *4 represent the 281 bp pBR322 *Alu* fragment containing the insertions G, GGGTACC, GGCTCGAGC, and GCGGAATTCCG, respectively, at the unique *Tth*III site. All insertions <63 bp were verified by sequencing the final construct.

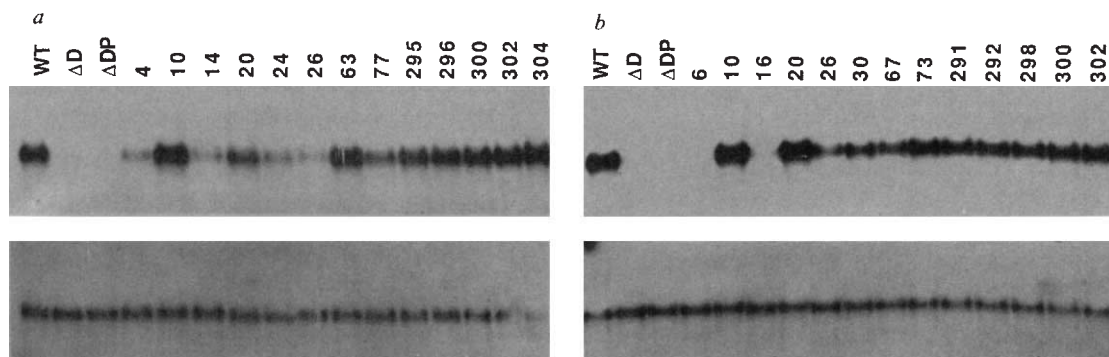
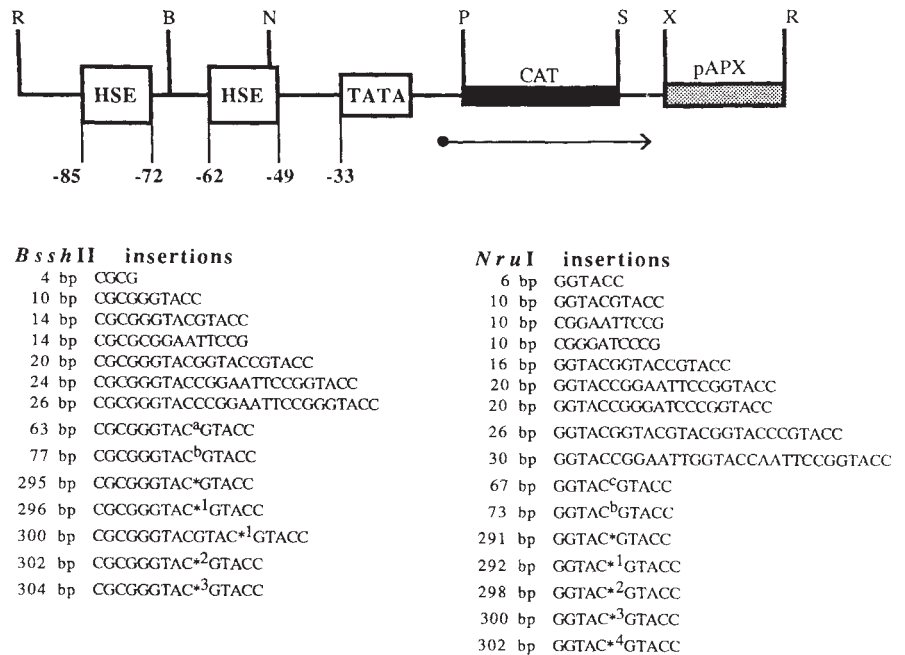
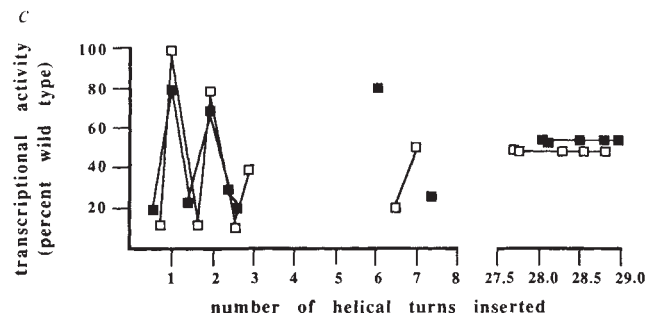


Fig. 2 Transcriptional activity of transfected *hsp70-cat* genes as a function of the length of DNA inserted at *Bss*II (a) or at *Nru*I (b). The autoradiograms shown in the top panels are of blots hybridized with an RNA probe specific for *hsp70-cat* transcripts. The autoradiograms shown in the bottom panels are of the same blots rehybridized with an RNA probe specific for transcripts produced by a cotransfected *hsp70-λ* gene. The *hsp70-λ* gene has a wild-type configuration of DNA control elements, providing an internal standard for determining the transcriptional activity of the *hsp70-cat* genes. The number of bp inserted into the various transfected *hsp70-cat* genes is given above the corresponding lanes. Other transfected genes and their lane assignments are as follows: WT, *hsp70-cat* gene with a wild-type configuration of control elements; ΔD, *hsp70-cat* deleted for the sequence upstream of -67 which includes the distal HSE; ΔDP, *hsp70-cat* deleted for the sequence upstream of -50 which includes both the distal and proximal HSEs. Amin *et al.*²⁵ found no reduction of *hsp70* expression (as measured by protein synthesis) upon the insertion of 4 bp of DNA at the *Bss*II site. It is not clear which of the several differences between their experiments and ours account for this discrepancy. c, Summary graph plotting the transcriptional activity of *hsp70-cat* genes with DNA insertions at *Bss*II (■) or at *Nru*I (□). Transcriptional activities are calculated as the ratio of the amount of transcripts produced by the *hsp70-cat* gene to the amount of transcripts produced by the *hsp70-λ* gene averaged over at least three independent experiments and normalized to the ratio of the amount of transcripts produced by the *hsp70-cat* gene having a wild-type configuration of DNA control elements to the amount of transcripts produced by the *hsp70-λ* gene. The transcriptional activity of a particular construct or of two constructs in which two different sequences of the same DNA length were inserted at the same site generally varied <20% from experiment to experiment.

Methods. *Drosophila* Schneider 2 cells ($\sim 50 \times 10^6$) were transfected with 1 μg of *hsp70-λ* DNA and 1 μg of *hsp70-cat* DNA in the presence of DEAE-dextran. Cells were harvested after 40–48 h and heat-shocked for 25 min at 37 °C. RNA was prepared immediately after the heat shock as described²⁶ and size-separated by gel electrophoresis (~ 5 μg per lane). Hybridizations were at 59 °C in 50% formamide, 10% PEG-6000, 7% SDS, 0.125 M sodium phosphate, 0.25 M NaCl and 0.001 M EDTA, pH 7.2. The relative intensities of autoradiographic bands were determined by comparing them with a series of bands whose relative intensities were known.



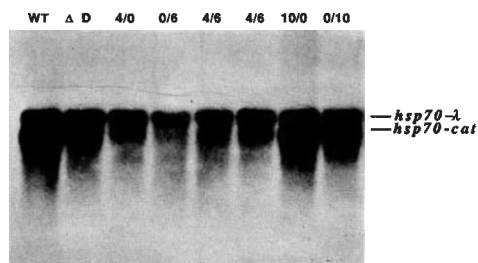


Fig. 3 Northern blot analysis of transcripts of *hsp70-cat* genes indicating a requirement for rotational alignment of at least two different protein-protein contacts involving at least three different DNA-protein binding sites. The blot was hybridized with RNA probes for transcripts of *hsp70-cat* and *hsp70-λ*. The numbers of bp inserted at the *Bss*II and *Nru*I sites of *hsp70-cat* are indicated to the left and right of the slash marks, respectively. Other constructs, abbreviations, and methods are described in the legend of Fig. 2.

as wild-type (compare the two lanes labelled 4/6 to lane WT). We conclude that for maximal transcription protein must interact with DNA in each of the three regions and that all three must be in unique rotational alignment.

In view of the cooperative binding of purified HSTF to the two HSEs *in vitro*, the two more distal regions of DNA-protein binding implied by our findings are almost certainly the proximal and distal binding sites of HSTF. The requirement for rotational alignment of these regions implies that HSTF molecules bound to them must form specific protein-protein contacts requiring unique rotational alignment. Such contact would presumably entail the bending or looping out of the DNA that lies between the bound sites^{1,7}.

Divergent homeo box proteins recognize similar DNA sequences in *Drosophila*

Timothy Hoey & Michael Levine

Department of Biological Sciences, Fairchild Center,
Columbia University, New York, New York 10027, USA

A member of a small group of genes in *Drosophila* that define the segmentation pattern of the early embryo¹⁻⁵ *even-skipped* (*eve*), which plays a key role in a network of interactions among segmentation genes. It appears to control morphogenesis by regulating the expression of the segmentation gene *engrailed* (*en*)^{3,4}, and by autoregulating its own expression (M. Frasch and M.L., in preparation). Here we show that these regulatory interactions could occur at the level of transcription as a full-length *eve* protein binds with high affinity to specific sequences located near the 5' ends of the *eve* and *en* genes. The *en* binding sites contain at least one copy of a 10-base pair consensus sequence: T-C-A-A-T-T-A-A-A-T. In contrast, the 5' *eve* binding sites are relatively G-C rich and do not share obvious similarities with the 10-base pair consensus sequence associated with *en*. Other homeo box proteins can recognize both classes of *eve* binding sites, lending support to the proposal that regulatory interactions among homeo box genes involve a competition of different homeo box proteins for similar cis regulatory sequences^{6,7}.

We have found that a full-length *eve* protein made in *Escherichia coli*⁸ binds to specific sequences near the *eve* and *en* genes using an immunoprecipitation assay⁹, in which cloned *en* and *eve* DNAs are incubated with *eve* protein, and protein/DNA complexes precipitated with polyclonal anti-*eve* antibodies. The highest affinity binding sites that were identified are summarized in Fig. 1a, b. Binding was detected to a 5' *en* fragment that maps between -949 base pairs (bp) and -708 bp

HSTF binding may extend to ~-40 and other proteins involved in transcription bind in the region of the TATA motif and the initiation site^{16,17,21}. The requirement for correct rotational alignment of the region 3' to the proximal HSE could, therefore, reflect contacts involving only HSTF molecules or contacts between HSTF and other proteins bound downstream.

We thank Vicki Corbin and Dale Dorsett for helpful discussions.

Received 2 February; accepted 14 March 1988.

1. Dunn, T., Hahn, S., Ogden, S. & Schleif, R. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5017-5020 (1984).
2. Hahn, S., Hendrickson, W. & Schleif, R. *J. molec. Biol.* **188**, 355-367 (1986).
3. Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A., Zenke, M. & Chambon, P. *Nature* **319**, 121-126 (1986).
4. Hochschild, A. H. & Ptashne, M. *Cell* **44**, 681-687 (1986).
5. Kraemer, H., Niemoller, M., Anouyal, M., Revet, B., von Wilcker-Bergman, B. & Muller-Hill, B. *EMBO J.* **6**, 1481-1491 (1987).
6. Griffith, J., Hochschild, A. & Ptashne, M. *Nature* **322**, 750-752 (1986).
7. Ptashne, M. *Nature* **322**, 697-701 (1986).
8. Pelham, H. R. B. & Bienz, M. *Eur. molec. Biol. Org. J.* **1**, 1473-1477 (1982).
9. Cohen, R. S. & Meselson, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5509-5513 (1984).
10. Dudler, R. & Travers, A. A. *Cell* **38**, 391-398 (1984).
11. Simon, J. A., Sutton, C. A., Lobell, R. B., Glaser, R. L. & Lis, J. T. *Cell* **40**, 805-817 (1985).
12. Simon, J. A. & Lis, J. T. *Nucleic Acids Res.* **15**, 2971-2988 (1987).
13. Pelham, H. R. B. *Trends Genet.* **1**, 31-35 (1985).
14. Amin, J., Mestril, R., Lawson, R., Klapper, H. & Voellmy, R. *Molec. cell. Biol.* **5**, 197-203 (1985).
15. Topol, J., Ruden, D. M. & Parker, C. S. *Cell* **42**, 527-537 (1985).
16. Wu, C. *Nature* **309**, 229-234; **311**, 81-84; **317**, 84-87 (1984).
17. Wu, C. *et al. Science* **238**, 1247-1253 (1987).
18. Parker, C. S. & Topol, J. *Cell* **37**, 273-283 (1984).
19. Wiederrecht, G., Shuey, D. J., Kibbe, W. A. & Parker, C. S. *Cell* **48**, 507-515 (1987).
20. Shuey, D. J. & Parker, C. S. *J. biol. Chem.* **261**, 7934-7940 (1986).
21. Parker, C. S. & Topol, J. *Cell* **37**, 273-283 (1984).
22. Shore, D. & Baldwin, R. L. *J. molec. Biol.* **170**, 957-982 (1983).
23. Horowitz, D. S. & Wang, J. C. *J. molec. Biol.* **173**, 75-91 (1984).
24. Cohen, R. S. & Meselson, M. *Cell* **43**, 737-746 (1985).
25. Amin, J., Mestril, R., Schiller, P., Dreano, M. & Voellmy, R. *Molec. cell. Biol.* **7**, 1055-1062 (1987).

upstream from the transcription start site. Two 5' *eve* fragments were found to bind, one mapping between -295 bp and -44 bp, and the other from -3.1 kilobases (kb) to -2.9 kb.

Binding sites were further characterized by DNaseI footprint experiments¹⁰. For the 5' *en* sites, a 240-bp *Clal*/*Hinf*I fragment (called fragment K according to the nomenclature of Desplan *et al.*⁶) was ³²P-labelled, incubated with increasing amounts of *eve* protein extract, and then partially digested with DNaseI (Fig. 2, lanes 1-4). Three different regions of protection are observed, designated k1, k2 and k3. Each of these sites contains at least one copy of a 10-bp consensus sequence: T-C-A-A-T-T-A-A-A-T (Fig. 1c). The k1 site contains 3 tandem copies of the consensus sequence and shows the highest relative binding in that k1 is protected at lower concentrations of protein as compared with the k2 and k3 sites (Fig. 2, lanes 2 and 3). For *eve*, DNaseI protection assays indicate the occurrence of two binding sites between -295 bp and -44 bp upstream from the start site (designated e4 and e5; Fig. 3, lanes 1-4). The sequences of these binding sites are relatively G-C rich and do not contain conserved copies of the 10-bp consensus sequence present within 5' *en* binding sites (see Fig. 1c). Nonetheless, these sites show an affinity for the *eve* protein which is comparable to that observed for the 5' *en* sites.

We have compared the binding activities of the *eve* protein with those encoded by *en*^{6,11}, the dorsal-ventral gene *zerknüllt* (*zen*)¹²⁻¹⁴, and the pair-rule gene *paired* (*prd*)^{15,16}. The 5' *en* fragment K was ³²P-labelled on the non-coding strand, separately incubated with each of the four protein extracts, and partially digested with DNaseI (Fig. 2). The three 5' *en* binding sites (k1, k2 and k3) are also protected by the *zen* protein, but are only weakly protected by the *en* and *prd* proteins. It is possible that the reduced binding observed for *en* and *prd* is due to lower inherent affinities of these proteins for the 5' *en* sites. However, an alternative explanation is that only a small fraction of these proteins is active, thereby reducing the overall specific activity of the *en* and *prd* protein extracts. We therefore

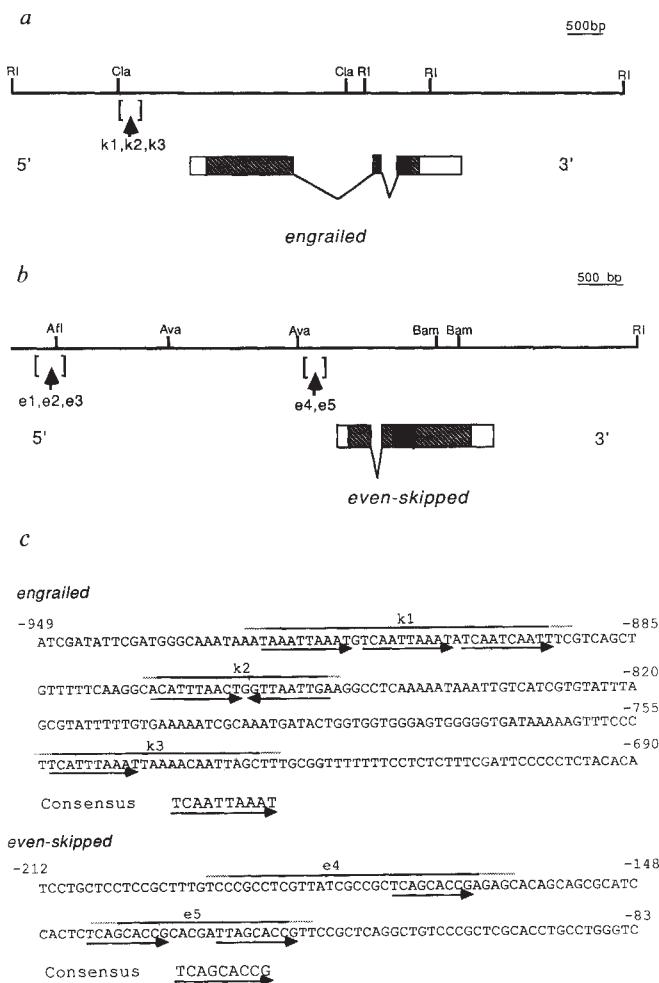


Fig. 1 Locations and sequences of *eve* protein binding sites. **a**, Map of the *en* gene¹¹ showing the location of the highest affinity *eve* protein binding sites. The brackets delineate a 240-bp fragment that contains a total of three binding sites (k1, k2, and k3), located between -949 bp and -708 bp upstream from the *en* transcription start site¹⁹. Exons and introns are indicated by rectangles and lines, respectively. The *en* homeo box is shaded black, and is interrupted by one of the introns. **b**, Map of the *eve* gene^{4,5} showing the highest affinity binding sites. There are three sites (e1, e2, and e3) between -3.1 kb and -2.9 kb, and two sites (e4, e5) between -295 bp and -44 bp upstream from the transcription start site. **c**, The uppermost sequence includes the three binding sites near *en*. The horizontal lines on top of the sequence indicate the boundaries of *eve* protein binding, based on DNaseI protection studies done with both strands of the fragment. The horizontal arrows below the sequence indicate conserved copies of a 10-bp consensus sequence (at least 7/10 matches). The bottom part of the figure shows the sequence of a proximal region of the *eve* promoter, which includes the e4 and e5 binding sites. The horizontal arrows indicate copies of a 9-bp consensus sequence (at least 8/9 matches).

Methods. Full-length *eve*, *zen*, *en*, and *paired* proteins were prepared with the T7 expression vector pAR3040, which contains the promoter and initiating methionine of the T7 gene⁸. An *Nde*I site was created at the translation initiation site for each coding sequence²⁰, and cloned into the unique *Nde*I site of the vector. Proteins were expressed in the bacterial strain BL21(DE3) as described in ref. 8. Induced cells were harvested by centrifugation and resuspended in 1/200 volume of buffer Z (100 mM KCl, 25 mM HEPES pH 7.8, 12.5 mM MgCl₂, 1 mM DTT, 0.1% NP-40, 20% glycerol, 1 mM PMSF, 2 mM benzamide, 5 µg ml⁻¹ leupeptin; 5 µg ml⁻¹ pepstatin A) containing 0.5 mg ml⁻¹ lysozyme. After sonication, the insoluble fraction was resuspended in buffer Z containing 4 M guanidine-HCl and incubated for 30 min at 4°C. The denaturant was removed by dialysis against buffer Z containing 1 M guanidine-HCl, followed by dialysis against buffer Z without guanidine-HCl.

decided to compare the relative binding of the different proteins to different sites using the k2 site as an internal standard.

Among the proteins that were tested, *zen* shows the highest relative affinity for the k1 site, and binds with an affinity ~25 times higher than that shown for k2. Binding of the *eve* protein to k1 is about twofold higher than binding to k2, whereas the *en* and *prd* proteins show about equal affinity for the k1 and k2 sites. Progressive binding of the *en* protein to the k1 site results in the appearance of several hypersensitive bands that are located between adjacent copies of the 10-bp consensus sequence (see asterisks, Fig. 2, lane 14). These hypersensitive sites are protected with increasing amounts of the *eve* and *zen* proteins. Different patterns of binding are also observed for the k2 site. A sequence of ~20 bp is protected by *eve* in the k2 region, whereas *zen* and *en* protect a sequence of only ~10 bp. The k2 site contains an inverted repeat of two copies of the 10-bp consensus sequence (see Fig. 1, c), and *eve* is able to protect both copies.

We have also compared the binding of the four homeo box proteins to the 5' *eve* e4 and e5 sites (Fig. 3). The *eve* protein binds with about equal affinity to the k2 and e4 sites, whereas each of the other proteins show at least fivefold lower relative binding to e4. Concentrations of the *zen*, *en*, and *prd* proteins that protect k2 fail to provide significant protection of the e4 site. The *zen* protein does, however, possess a low affinity for e4 as increasing concentrations of the protein result in the appearance of hypersensitive bands within the e4 site (lane 9, Fig. 3). The *paired* protein shows the highest relative binding to the e5 site, in that the highest concentrations of the protein that were assayed give about equal protection of k2 and e5 (compare lane 19, Fig. 2 with lane 19, Fig. 3). Interestingly, *eve* and *prd* do not protect exactly the same region within e5, and appear to protect overlapping sequences (compare lane 4 with lane 19, Fig. 3).

Here we have shown that a full-length *eve* protein binds with about equal affinity to two different classes of binding sites: (1) A-T rich sites located near the 5' end of *en* and (2) G-C rich sites located near *eve*. Past *in vivo* circuitry studies indicate that *eve* participates in the activation of *en* expression, and in some way influences its own expression (refs 3, 4; M. Frasch and M.L., in preparation). The occurrence of high affinity *eve* protein binding sites near the *en* and *eve* genes is consistent with the possibility that one or both of these interactions occur at the level of transcription. Among the four homeo box proteins that we have examined, *eve* is unique in showing almost the same relative binding to the 5' *en* k1 site and the 5' *eve* e4 site. In contrast, the other proteins, particularly *zen*, show a preference for k1. Using binding to the k2 site as an internal control, *zen* shows a relative affinity ~100 times higher for k1 than for e4. Thus, it would appear that different homeo box proteins recognize a broad range of DNA sequences, and that preferences for specific subsets of these sequences could vary among the different proteins according to the sequence of the homeo domain and/or some other critical recognition region within these proteins. Pairwise sequence comparisons show that no two of the proteins share >52% amino acid identity within the homeo domain, and they share no significant similarity outside the homeo domain.

There are at least two possible mechanisms for regulatory interactions among homeo box genes. In a combinatorial model, each homeo box protein recognizes a qualitatively different DNA sequence. The 'on/off' condition of a particular target gene could depend on what combination of homeo box proteins is bound to the diverse *cis* regulatory sequences associated with that gene. Such a mechanism is similar to the proposed regulation of cell type-specific genes in yeast by $\alpha 1$ and $\alpha 2$ ¹⁷. Alternatively, it is possible that all homeo box proteins bind to the same limited set of DNA binding sequences with different affinities. In this latter model, different homeo box proteins 'compete' for binding to a specific *cis* regulatory element, and

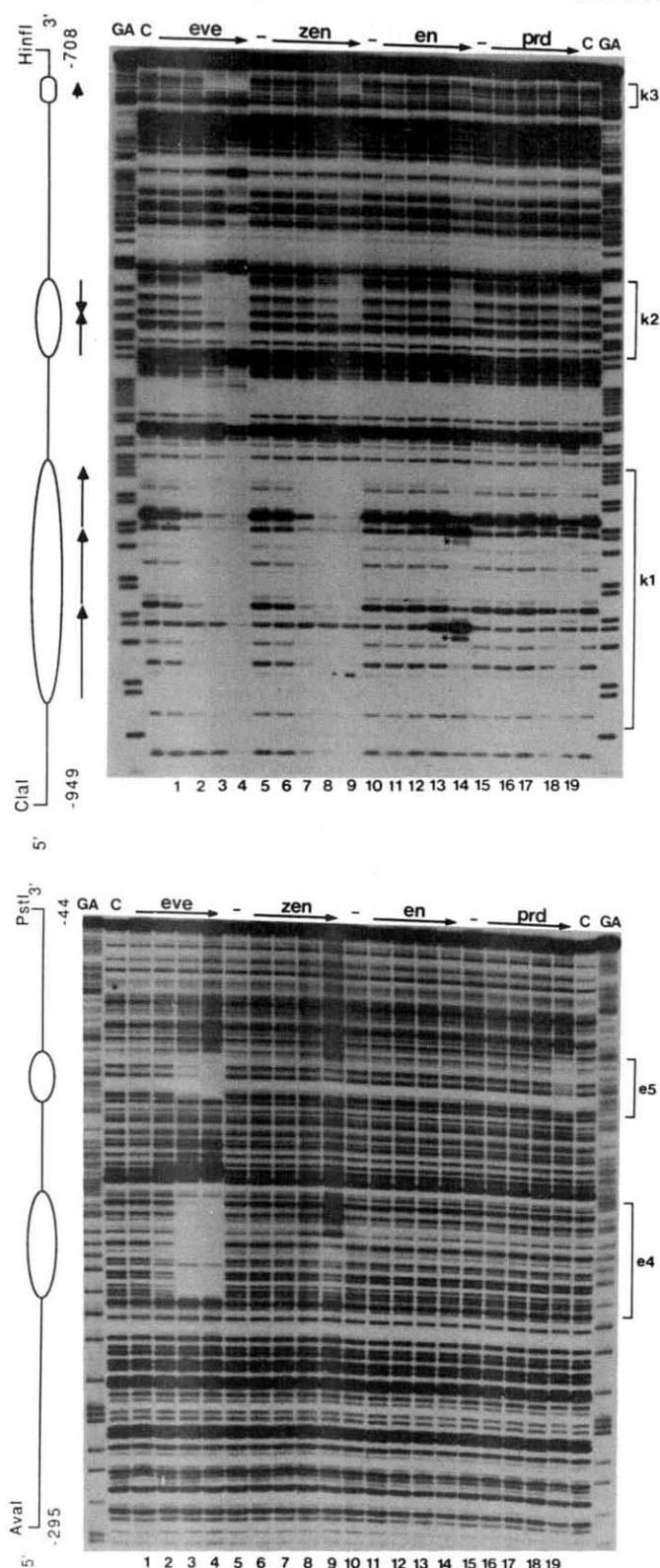


Fig. 2 Comparative binding of homeo box proteins to 5' *en* sequences. The 240-bp *en* fragment K was 32 P-labelled at the *Cla*I site (see Fig. 1a) and incubated with increasing amounts of *eve*, *zen*, *en*, and *prd* protein extracts. Lanes 1–4, 0.04, 0.2, 1 and 5 μ g respectively of *eve* protein extract. There are three regions that are protected from digestion with DNaseI, labelled k1, k2 and k3. The diagram to the left of the autoradiogram represents the binding of the protein to each of these sites. The vertical arrows indicate the locations and orientations of a 10-bp consensus sequence within each site (see Fig. 1c). Note that high concentrations of *eve* protein give weak binding in regions located between the k1 and k2 sites, and between k2 and k3. However, these sites have <20-fold the affinity of the k2 site. Lanes 6–9, 0.04, 0.2, 1 and 5 μ g respectively of *zen* protein extract. Lanes 11–14, 0.08, 0.4, 2 and 10 μ g respectively of *en* protein extract. Note the appearance of hypersensitive bands (denoted by an asterisk) with increasing amounts of *en* protein. These sites are located between adjacent copies of the 10-bp consensus sequence. Lanes 16–19, 0.08, 0.4, 2 and 10 μ g respectively of *prd* protein extract. GA, the deoxyguanosine + deoxyadenosine sequence of fragment K; C, negative controls done with 5 μ g protein extract from cells containing the T7 expression vector without the homeo box cDNA inserts. Lanes labelled (–) indicate DNaseI digestion of fragment K without added protein. **Methods.** DNaseI protection assays were done essentially as described²¹. Binding reactions were done with 2–5 ng of 32 P-labelled DNA and 5 μ g of sonicated calf thymus DNA in 50 μ l for 30–45 min on ice. The composition of the binding reaction, including the buffer components of the extract, was 110 mM KCl, 47.5 mM HEPES pH 7.8, 13.75 mM MgCl₂, 0.05 mM EDTA, 1 mM DTT, 17% glycerol, and 0.05% NP-40 detergent. After binding, 50 μ l 10 mM MgCl₂/5 mM CaCl₂ were added to the reaction, followed by 5 μ l of freshly diluted DNaseI (Worthington) at a final concentration of 10 μ g ml⁻¹. DNaseI digestion was stopped after 5 min on ice by addition of 90 μ l 1% SDS, 20 mM EDTA, 200 mM KCl, 250 μ g ml⁻¹ yeast transfer RNA. The samples were extracted twice with phenol-chloroform (1:1), ethanol precipitated, and electrophoresed in an 8% polyacrylamide/7.5 M urea gel.

Fig. 3 Comparative binding to 5' *eve* sequences. A 250-bp fragment from –295 bp to –44 bp upstream from the *eve* transcription start site was 32 P-labelled at an *Ava*I site (see Fig. 1b, c), and incubated with increasing amounts of each of the four protein extracts, as for the experiment shown in Fig. 2. Lanes 1–4 show increasing amounts of the *eve* protein. Two regions of protection, e4 and e5, are observed. The diagram to the left of the autoradiogram represents the binding of the protein to these sites. Lanes 6–9 were done with increasing amounts of the *zen* protein. High concentrations of the protein result in the appearance of hypersensitive bands near the e4 and e5 sites (lane 9). Lanes 11–14 were done with increasing amounts of *en* protein. Even high concentrations fail to protect either the e4 or e5 site. But the amount of *en* protein used in lane 14 is sufficient to give protection of the 5' *en* k1 site (see lane 14 of Fig. 2). Lanes 16–19 used increasing amounts of *prd* protein. Note that high concentrations of the protein protect the e5 site but not e4 (lane 19). The region protected by the *prd* protein extensively overlaps, but does not exactly coincide with, the e5 footprint observed for the *eve* protein (compare lanes 4 and 19).

the 'on/off' condition of a target gene depends on which homeo box protein has the highest affinity, and also on the intracellular concentration of the various proteins^{6,7}. This type of mechanism is analogous to the regulatory interactions of lambda repressor and cro proteins¹⁸. In their extreme forms, probably neither of these models is adequate to account for regulatory interactions among homeo box genes. It is possible that both mechanisms are actually used *in vivo*. The binding of *eve*, *zen*, *en* and *prd*

proteins to 5' *en* sites supports a competition model, whereas the more selective binding of the *eve* protein to the 5' *eve* sites is consistent with a combinatorial mechanism.

We thank Tom Kornberg for the full-length *en* cDNA and Markus Noll for the full-length *prd* cDNA. We also thank Claude Desplan and Pat O'Farrell for *en* genomic DNAs and for providing the unpublished sequence of fragment K, and C. Desplan for advice and encouragement. We thank Yoshio

Takagaki for pointing out the consensus sequence within the e4 and e5 sites. This work was funded by grants from the NIH and the Searle Scholars' Program.

Received 7 January 1988; accepted 7 March 1988.

1. Nusslein-Volhard, C. & Wieschaus, E. *Nature* **287**, 795-801 (1980).
2. Nusslein-Volhard, C., Kluding, H. & Jurgens, G. *Cold Spring Harbor Symp. Quant. Biol.* **50**, 145-154 (1985).
3. Harding, K., Rushlow, C., Doyle, H. J., Hoey, T. & Levine, M. *Science* **233**, 953-959 (1986).
4. Macdonald, P. M., Ingham, P. W. & Struhl, G. *Cell* **47**, 721-734 (1986).
5. Frasch, M., Hoey, T., Rushlow, C., Doyle, H. & Levine, M. *EMBO J.* **6**, 749-759 (1987).
6. Desplan, C., Theis, J. & O'Farrell, P. H. *Nature* **318**, 630-635 (1985).
7. Harding, K., Wedeen, C., McGinnis, W. & Levine, M. *Science* **229**, 1236-1242 (1985).
8. Studier, F. W. & Moffatt, B. A. *J. molec. Biol.* **189**, 113-130 (1986).
9. McKay, R. J. *J. molec. Biol.* **145**, 471-488 (1981).
10. Galas, D. & Schmitz, A. *Nucleic Acids Res.* **5**, 3157-3170 (1978).
11. Poole, S. J., Kauvar, L. M., Drees, B. & Kornberg, T. *Cell* **40**, 37-43 (1985).
12. Wakimoto, B. T., Turner, F. R. & Kaufman, T. C. *Dev. Biol.* **102**, 147-172 (1984).
13. Doyle, H. J., Harding, K., Hoey, T. & Levine, M. *Nature* **323**, 76-79 (1986).
14. Rushlow, C., Doyle, H., Hoey, T. & Levine, M. *Genes & Dev.* **1**, 1268-1279 (1987).
15. Kilcherr, F., Baumgartner, S., Bopp, D., Frei, E. & Noll, M. *Nature* **313**, 284-289 (1985).
16. Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. & Noll, M. *Cell* **47**, 735-746 (1986).
17. Miller, A. M., MacKay, V. L. & Nasmyth, K. *Nature* **314**, 598-603 (1985).
18. Ptashne, M. *et al. Cell* **19**, 1-11 (1980).
19. Drees, B. *et al. EMBO J.* **6**, 2803-2809 (1987).
20. Zoller, M. J. & Smith, M. *DNA* **3**, 479-488 (1984).
21. Heberlein, U., England, B. & Tjian, R. *Cell* **41**, 965-977 (1985).

Uncoupling of the recombination and topoisomerase activities of the $\gamma\delta$ resolvase by a mutation at the crossover point

Eileen Falvey*, Graham F. Hatfull & Nigel D. F. Grindley†

Department of Molecular Biophysics and Biochemistry, Yale University Medical School, PO Box 3333, 333 Cedar St., New Haven, Connecticut 06510, USA

In several well-characterized site-specific recombination systems it has been shown that, for efficient recombination, the two recombining sites must have identical DNA sequences across the region between the staggered points of exchange. The precise DNA sequence of this overlap region, however, appears to be of little importance¹⁻⁶ (with the exception of one position in the *loxP* site of bacteriophage P1 (ref. 6)). In this report we characterize a mutant recombination site for the site-specific recombination enzyme $\gamma\delta$ resolvase (encoded by the $\gamma\delta$ transposon), in which the dinucleotide at the crossover point is changed from AT to CT. Our results indicate that identity of the two overlap regions is not sufficient for recombination. Although resolvase binds normally to the mutant site and induces the structural deformation characteristic of the wild-type recombination site, catalysis at the crossover point (cutting and rejoining of DNA strands) is effectively limited to just one of the two strands, allowing resolvase to act as a topoisomerase but not as a recombinational enzyme.

$\gamma\delta$ resolvase is a protein of relative molecular mass (M_r) 21,000 encoded by the transposon $\gamma\delta$, a member of the Tn3 family of bacterial transposons⁷. It catalyses a conservative site-specific recombination event between two copies of its cognate recombination site, *res*, present on a single replicon. This reaction has been accomplished *in vitro* in a simple buffer with Mg^{2+} and resolvase, producing a pair of catenated circles from a superhelical substrate⁸. In reactions carried out in the absence of Mg^{2+} , linear half molecules are formed by cleavage of substrate molecules at the two recombination sites. Analysis of these *in vitro* products has established that strand exchange proceeds

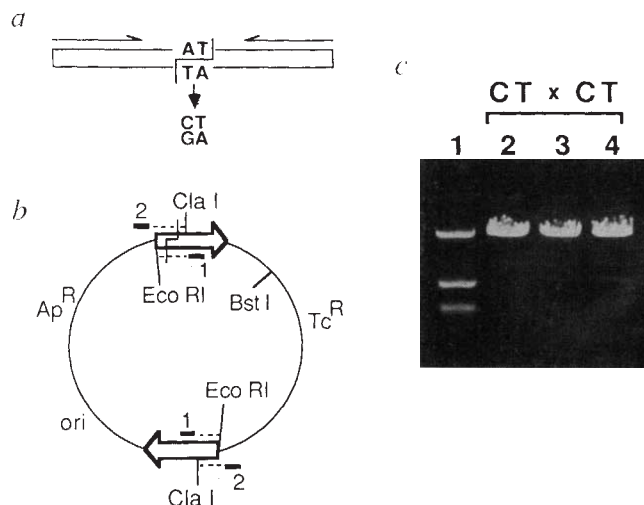


Fig. 1 A mutation within the overlap region inhibits resolution *in vitro*. **a**, Site I of *res*, showing the 2 base pair (bp) wild-type and mutant overlap regions. The inverted arrows represent the resolvase binding segment. **b**, Resolution substrate, pGH448, is a cointegrate analogue containing two *res* sites (open arrows) with the central CT dinucleotide (its construction will be described elsewhere). The positions of relevant restriction enzyme sites, as well as positions of the two oligonucleotides used in the experiment in Fig. 4, are shown. Oligo 1, 5'-GACACATTAACAGCACTG-3', anneals to the site I-site II spacer region. Oligo 2, 5'-GTATCAGAGGCCCT-3', anneals to pBR322 sequences to the left of the *res* insert. **c**, *In vitro* resolution of cointegrates. Lane 1, wild-type substrate (pNG176) with 0.9 μ M resolvase; lanes 2-4, pGH448, with no resolvase (lane 2), 0.9 μ M resolvase (lane 3), and 9 μ M resolvase (lane 4).

Methods. Standard resolution reactions were carried out as described⁸. Reactions (30 μ l) contained ~10 nM plasmid DNA, and resolvase at the concentration specified above. Samples were digested with *Bst*I and run on a 1% agarose gel. Unresolved DNA migrates as a 5.1 kilobase (kb) linear fragment; products of resolution appear as a 2.4 kb linear fragment and a faster migrating 2.7 kb circular molecule (see lane 1; faintly discernible in lanes 3 and 4).

through a staggered cut, generating a two base 3' overlap at the recombination crossover point⁹. Covalent linkage of resolvase to the 5' phosphates conserves the phosphodiester bond energy.

We have examined the properties of a *res* site in which the central dinucleotide (the overlap region) of binding site I has been mutated from the wild-type AT to CT (see Fig. 1a). A cointegrate with two copies of the mutant *res*, depicted schematically in Fig. 1b, shows a severe defect in resolution. Very little recombination was seen *in vitro*, even at a concentration of resolvase that was 10-fold higher than necessary for complete resolution of a wild-type substrate (Fig. 1c, lanes 3 and 4). Clearly, the sequence of the overlap region is an important feature of the *res* site.

What is the basis for the recombinational defect of the mutant crossover site? Early steps, the binding of resolvase to the mutant site I and the induction of the structural deformation, appear to be indistinguishable from a wild-type substrate. Figure 2a shows that DNA fragments containing mutant and wild-type forms of site I give rise to equivalent amounts of resolvase-DNA complexes at several different resolvase concentrations. Moreover, when complexes are footprinted with the intercalating reagent methidiumpropyl EDTA.Fe(II) (MPE), both mutant and wild-type substrates give the same enhanced cleavage at the centre of the binding site (Fig. 2b). As we have shown earlier, this enhanced cleavage appears to detect a resolvase-dependent distortion of the DNA structure (possibly a kink) at the crossover point^{11,12}.

If the mutant inhibits a later stage in the reaction pathway,

* Present address: Department of Molecular Biology, University of Geneva, 1211 Geneva 4, Switzerland.

† To whom correspondence should be addressed.

Fig. 2 *a*, Autoradiogram of a polyacrylamide gel used to assay the formation of complexes between resolvase and either mutant or wild-type site I DNA fragments. Each binding reaction contains both mutant and wild-type site I DNA fragments. Protein was added as follows: no resolvase (lane 1); 5 nM resolvase (lane 2), 17 nM resolvase (lane 3), 50 nM resolvase (lane 4). DNA fragments were obtained by primer extension of M13 DNA containing site I or mutant site I. This version of the CT mutant has M13 sequences flanking site I. Sizes of fragments are shown in bp. *b*, Comparison of wild-type (lanes 2–5) and mutant site I (lanes 6–9) by MPE.Fe(II) footprinting. Resolvase was added as follows: no resolvase (lanes 2 and 6), 5 nM resolvase (lanes 3 and 7), 50 nM resolvase (lanes 4 and 8), 500 nM resolvase (lanes 5 and 9). Lane 1 is a dideoxythymidine sequencing track. Arrows mark positions of enhanced MPE.Fe(II) cleavage.

Methods. Fragments for (*a*) were prepared by annealing universal primer to M13 DNA containing site I inserts, and extending with Klenow, using 50 μ M each of dCTP, dGTP and dTTP, and 5 μ M dATP (30:1 dATP: [α - 32 P]dATP) for 15 min at 25 °C. This DNA was then restricted with *Eco*RI and *Pvu*II, which released site I on a 224 bp fragment. A wild-type internal standard was included, made by primer extension of an M13 clone of wild-type site I sequence and restriction with *Eco*RI and *Hind*III, which released a 46 bp fragment. Binding conditions and nondenaturing gel electrophoresis of complexes have been described²⁰. DNA fragments were added to a final concentration of ~0.1 nM (fragment). DNA fragments for (*b*) were prepared by primer extension of the same M13 DNAs as in (*a*) using 5' end-labelled primer and 25 μ M dNTPs. Footprinting reactions were as described¹¹.

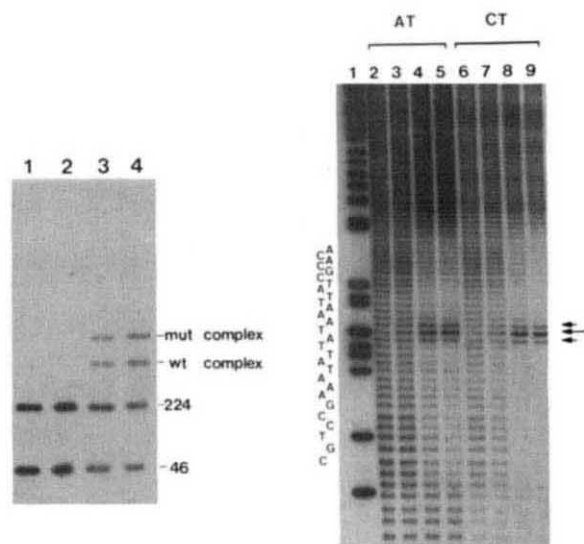
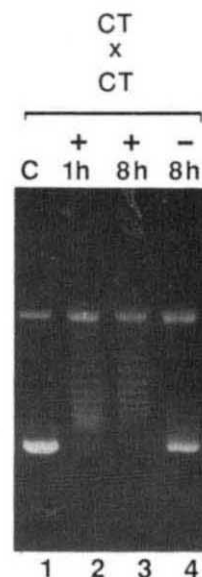


Fig. 3 Superhelical relaxation of the CT mutant cointegrate *in vitro*. Resolvase reactions were carried out with Mg^{2+} , for 1 h (lane 2) or 8 h (lane 3), or without Mg^{2+} for 8 h (lane 4). DNA in lane 1 was incubated without resolvase. The fastest migrating material is superhelical substrate DNA, the slowest form is nicked substrate; partially relaxed topoisomers migrate in between.

Methods. Resolvase reactions were as described⁸. Reactions contained 10 nM plasmid DNA and 900 nM resolvase in 30 μ l total volume. After proteinase K treatment, DNA was phenol-extracted, ether-extracted, and a portion was loaded on a 1% agarose gel. The gel was run at 2V cm^{-1} for 11 h in 40 mM Tris-acetate, pH 7.9, 5 mM Na acetate, 1 mM EDTA, and visualized by staining with ethidium bromide.



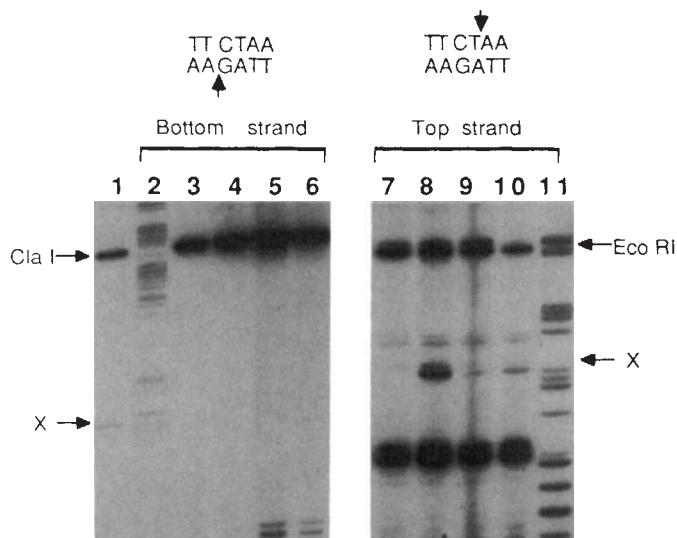
intermediates or side products might accumulate. Indeed, resolvase catalysed substantial relaxation of the mutant substrate into topoisomers of reduced negative superhelicity (Fig. 3, lanes 2 and 3). In addition a portion of the substrate was converted into a nicked form. Relaxation of superhelical DNA by resolvase is known to require two copies of *res* in the same orientation¹³. Presumably, formation of an appropriate synaptic complex activates the recombinational potential of resolvase. Because the mutant cointegrate is a good substrate for relaxation (and a single mutant site is inactive), we conclude it forms the synaptic complex. Although superhelical relaxation of the mutant substrate was inhibited in the absence of Mg^{2+} , nicking of the substrate was not (or was even increased slightly).

To determine whether the formation of nicked molecules was site-specific, we performed primer extension assays with two synthetic oligonucleotide primers (shown schematically in Fig. 1b). As can be seen in Fig. 4 (lanes 7–11) extension of the primer annealed to the top strand yielded products that corresponded

to substrate nicked at the position 5'-TTCT⁺AA in the presence and absence of Mg^{2+} . By contrast, very little cutting was detected on the bottom strand of the mutant substrate (AA₁GATT-5'; Fig. 4, lanes 3–6), although a faint band was detectable upon prolonged exposure. Densitometric measurements determined that, while as much as 35% of the reaction products were nicked at the site of strand exchange on the top strand, the level of nicking on the bottom strand was less than 1%. In similar experiments with a wild-type *res* cointegrate substrate, efficient cleavage of both strands was detected in reactions without Mg^{2+} (30–40% of substrate molecules) but less than 1% of the molecules were cleaved on either strand in reactions with Mg^{2+} (data not shown). The accumulation of nicked products from the mutant substrate early in the reaction, even in the presence of Mg^{2+} , indicates that rejoining of the cleaved single strand does not occur efficiently. However, after 8 h (Fig. 4, lane 9) the amount of specifically nicked product was significantly reduced, suggesting that the nicks apparent at early times are

Fig. 4 Detection of nicks at the mutant crossover site by primer extension analysis of *in vitro* resolution products. Synthesis primed by oligo 1 (see Fig. 1b) was used to detect nicking on the top strand, between the wild-type base pairs in the sequence 5'-TTCT⁺AA (lanes 7-11). Synthesis primed by oligo 2 was used to detect nicking on the bottom strand, adjacent to the mutant base pair in the sequence AA₊GATT-5' (lanes 2-6). Template DNAs were derived from the resolution reactions analysed in Fig. 3 as follows: unresolved substrate DNA (lanes 3 and 7), the reactions in the presence of Mg²⁺ for 1 h (lanes 4 and 8) and 8 h (lanes 5 and 9), and the 8 hr reaction without Mg²⁺ (lanes 6 and 10). A nick at the crossover site generates products that migrate at the positions marked X. Un-nicked template generates products that terminate at the *Cla*I site (bottom strand) or the *Eco*RI site (top strand) which lie just beyond the crossover site from the annealed primer (see Fig. 1b). Lane 1 is a control lane to show the position of the product generated by a bottom strand nick (the template DNA was from a reaction with resolvase and wild-type substrate DNA in the absence of Mg²⁺). Lanes 2 and 11 are dideoxythymidine sequencing reactions with untreated template DNA.

Methods. Portions of the resolution reactions analysed in Fig. 3 were digested with either *Eco*RI (for hybridization with oligo 1, lanes 1-6) or *Cla*I (for oligo 2, lanes 7-11). DNA was ethanol-precipitated and redissolved in 16 µl water. The DNA was denatured by adding 4 µl 1 M NaOH and incubating the sample for 5 min at 25 °C. Samples were neutralized by addition of 2 µl of 2 M ammonium acetate, pH 4.5, and the DNA was ethanol-precipitated. It was then dissolved in 10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, and 0.1 pmol 5'-labelled oligonucleotide primer was added. Mixtures were incubated at 42 °C for 30 min to allow annealing. To 4 µl annealed DNA, 2 µl of a mix containing 250 µM dNTPs, 10 mM DTT, and 1 µl Klenow was added and the reaction was incubated at 37 °C for 15 min. Samples were denatured and loaded on a 10% polyacrylamide, 8 M urea sequencing gel.



transient intermediates of resolvase-mediated topoisomerase activity. These experiments show that the action of resolvase at the mutant site is highly asymmetric, with almost complete inhibition of its phosphoryltransferase activity on the bottom strand of the DNA, adjacent to the mutant base pair. (Alternatively, cutting of both strands occurs equally, but rejoining of the top strand is inhibited. This is unlikely as double-strand cutting is not observed in the reactions lacking Mg²⁺.)

There are two possible explanations for the biochemical defect. First, resolvase could make specific contacts with the bases of the overlap region which are important in the recombinational process (although not involved in determination of binding affinity—see Fig. 2a). Second, despite the similarity in MPE sensitivity, the base sequence might affect the structure of the crossover site, (perhaps by displacing the cuttable phosphodiester bond relative to the active site of resolvase), thus affecting the relative rates of cleavage of the two DNA strands.

The asymmetric action of resolvase at the mutant crossover site suggests that the topoisomerase activity observed with this substrate proceeds by nicking and closing only the top strand. This does not necessarily imply, however, that recombination also occurs by sequential single strand exchanges (as appears to be the case with site-specific recombination mediated by the phage λ Integrase^{14,15}). Several lines of evidence favour a mechanism for resolvase that involves concerted cutting of both strands at both crossover points. The double strand cutting of both *res* sites in the absence of Mg²⁺ supports such a mechanism, as do recent topological analyses of recombination by the Tn3 resolvase¹⁶ and the related DNA-invertase, Gin¹⁷. It has been suggested that the type I topoisomerase activity of resolvase

supports a recombinational mechanism that involves single strand exchanges¹³. We would argue, however, that the synaptic complex, formation of which is necessary for initiation of topoisomerase activity, must dissociate before completion of the process. Without such dissociation, rejoining of broken duplexes in the parental (that is, non-recombinant) configuration would be equivalent to two rounds of reiterative recombination which is known to produce a four-noded knot¹⁸. Thus, we suggest that topoisomerase activity of resolvase is seen only when the rejoining of DNA strands becomes uncoupled from their cleavage. Such uncoupling is likely to result from any perturbations of resolvase, its substrate DNA, or their interactions, which reduce the stability or recombinational efficiency of the synaptic complex (and indeed the Tn21 resolvase acts as an efficient topoisomerase, but a poor recombinase, at low ionic strength¹⁹). Once dissociation of the synaptic complex occurs, topoisomerase activity will result from rotations of broken ends at a crossover site in either of the following ways: first, if both strands are cut, one duplex end rotates 360° relative to the other about the helix axis, and is then rejoined to its partner; second, if only one strand is cut, one free end rotates 360° about the unbroken strand and the nick is sealed. Both sets of events change the initial substrate linking number by one (that is, a type I topoisomerase activity) even though in the first case both strands are broken. Although relaxation of our mutant substrate almost certainly occurs by the second pathway, relaxation of a wild-type substrate could occur by either pathway, and perhaps does.

This work was supported by a grant from the NIH.

Received 7 January; accepted 10 March 1988.

- Weisberg, R. A., Enquist, L. W., Foeller, C. & Landy, A. *J. molec. Biol.* **170**, 319-342 (1983).
- Bauer, C. E., Gardner, J. E. & Gumpert, R. I. *J. molec. Biol.* **181**, 187-197 (1985).
- de Massy, B., Studier, F. W., Dorgai, L., Appelbaum, E. & Weisberg, R. A. *Cold Spring Harb. Symp. quant. Biol.* **49**, 715-726 (1984).
- Hoess, R. H., Wierzbicki, A. & Abremski, K. *Nucleic Acids Res.* **14**, 2287-2300 (1986).
- Johnson, R. C. & Simon, M. I. *Cell* **41**, 781-791 (1985).
- Iida, S. & Hiestand-Nauer, R. *Cell* **45**, 71-79 (1986).
- Grindley, N. D. F. & Reed, R. R. *Rev. Biochem.* **54**, 863-896 (1985).
- Reed, R. R. *Cell* **25**, 713-719 (1981).
- Reed, R. R. & Grindley, N. D. F. *Cell* **25**, 721-728 (1981).

- Hatfull, G. F., Noble, S. M. & Grindley, N. D. F. *Cell* **49**, 103-110 (1987).
- Salvo, J. J. & Grindley, N. D. F. *Nucleic Acids Res.* **15**, 9771-9779 (1987).
- Krasnow, M. A. & Cozzarelli, N. R. *Cell* **32**, 1313-1324 (1983).
- Nash, H. A., Bauer, C. E. & Gardner, J. F. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4049-4053 (1987).
- Nunes-Duby, S. E., Matsumoto, L. & Landy, A. *Cell* **50**, 779-788 (1987).
- Boocock, M. R., Brown, J. L. & Sherratt, D. J. in *DNA Replication and Recombination* (eds Kelley, T. J. & McMacken, R.) 703-718 (Liss, New York, 1987).
- Kanaar, R., van de Putte, P. & Cozzarelli, N. R. *Proc. natn. Acad. Sci. U.S.A.* **85**, 752-756 (1988).
- Krasnow, M. A. *et al. Nature* **304**, 559-560 (1983).
- Castell, S. E., Jordan, S. L. & Halford, S. E. *Nucleic Acids Res.* **14**, 7213-7226 (1986).
- Hatfull, G. F. & Grindley, N. D. F. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5429-5433 (1986).

GUIDE TO AUTHORS

Please follow these guidelines so that your manuscript may be handled expeditiously.

Nature is an international journal covering all the sciences. Contributors should therefore bear in mind those readers for whom English is a second language and those who work in other fields. Please write clearly and simply, avoiding unnecessary technical terminology. *Nature's* staff will edit manuscripts to those ends if necessary. Contributors should check their proofs carefully.

Because of the competition for space, many of the papers submitted for publication cannot be accepted. For this reason, and because brevity is a great assistance to readers, papers should be as brief as is consistent with intelligibility. Please note that one printed page of *Nature*, without diagrams or other interruptions of the text, has fewer than 1,300 words.

CATEGORIES OF PAPER

Review Articles survey recent developments in a field. Most are commissioned, but suggestions are welcome in the form of a one-page synopsis addressed to the Reviews Coordinator. Length is negotiable in advance but must not exceed six pages of *Nature*.

Articles are research reports whose conclusions are of general interest and which are sufficiently rounded to be a substantial advance in understanding. They should not have more than 3,000 words of text or more than six display items (figures and tables) and should not occupy more than five pages of *Nature*.

Articles should be accompanied by a heading of 50–80 words written to advertise their contents in general terms, to which editors will pay particular attention. A heading (printed in italic type) is not an abstract and should not usually contain numbers or measurements. The study should be introduced in more detail in the first two or three paragraphs, which should also briefly summarize its results and implications.

Articles may contain a few subheadings of two or three words. The meaning of the text should not depend on the subheadings, whose function is to break up the text and to point to what follows. There should be fewer than 50 references.

Letters to *Nature* are short reports of outstanding novel findings whose implications are general and important enough to be of interest to those outside the field. Letters should not have more than 1,000 words of text or more than four display items and should not occupy more than two pages of *Nature*. The first paragraph should describe, in not more than 150 words, the origins and chief conclusions of the study. Letters should not have subheadings or more than 30 references.

Commentary articles deal with issues in, or arising from, research that are also of interest to readers outside research. Some are commissioned, most are unsolicited. They are normally between one and four pages of *Nature* in length.

News and Views articles are intended to inform non-specialist readers about a recently published advance. Suggestions should be made to the News and Views Editor. Illustrations are welcome. Proposals for meeting reports should be agreed in advance.

Scientific Correspondence is for the discussion of scientific matters, including contributions published in *Nature*. Priority is given to contributions of less than 500 words and five references. Figures are welcome.

Submission. All manuscripts may be submitted to either London or Washington. They should not be addressed to editors by name. Manuscripts or proofs sent by air courier to London should be declared as 'manuscripts' and 'value \$5' to prevent the imposition of import duty and value-added tax (VAT).

GENERAL

All contributions submitted for publication in *Nature* should conform with these rules.

Manuscripts should be typed, double-spaced, with a good-quality printer, on one side of the paper only. Four copies are required, each accompanied by lettered artwork. Four copies of half-tones should be included. Reference lists, figure legends and so on should be on separate sheets, all of which should be double-spaced and numbered. Copies of relevant manuscripts in press or submitted for publication elsewhere should be included, clearly marked as such. Revised and resubmitted manuscripts should also be clearly marked as such and labelled with their reference numbers.

Titles should say what the paper is about with the minimum of technical terminology and in fewer than 80 characters. Authors should avoid active verbs, numerical values, abbreviations and punctuation and should include one or two key words for indexing purposes.

Artwork should be marked individually and clearly with the author's name, figure number and, when known, manuscript reference number. Original artwork should be unlettered. Ideally, no figure should be larger than 28 by 22 cm. Figures with several parts are permitted only if the parts are closely related, either experimentally or logically. Suggestions for cover illustrations, with captions, are welcome. Original artwork (and one copy of the manuscript) will be returned when a manuscript cannot be published.

Colour Artwork. A charge of £500 a page is made for 4-colour figures. Inability to meet these costs will not prevent the publication of essential colour figures if the circumstances are explained.

Figure legends must not exceed 300 words and ideally should be much shorter, and should use telegraphic form wherever possible. The figure should be described first, then, briefly, the method. Reference to a method described elsewhere is preferable to a full description. Methods should not be described in detail in the text.

References should be numbered sequentially as they appear in the text, followed by those in tables and finally those in the figure legends. Reference numbers apply only to papers published or in the press, and a different number should be given to each paper cited. All other forms of reference (including unrefereed abstracts) should be included in the text as personal communication, manuscript in preparation or preprint (with number and institution where appropriate). Text should not be included in the reference list. References should be abbreviated according to the *World List of Scientific Periodicals* (fourth edition, Butterworth, London, 1963–65). The first and last page numbers should be cited. Reference to books should clearly indicate the publisher and the date and place of publication.

Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided as much as possible and, when used, defined. Editors will shorten words if necessary. In case of doubt, SI units should be used.

Footnotes should not be used except for changed addresses or to identify the corresponding author if different from the first-named.

Acknowledgements should be brief. Grant numbers and contribution numbers are not allowed.

Isotope-edited NMR spectroscopy

S.W. Fesik

Detailed information on the structure of large enzyme/inhibitor complexes can be obtained by simplifying proton NMR spectra through isotope-editing.

NMR spectroscopy is a powerful tool for determining the three-dimensional structure of molecules or molecular complexes of M_r less than 15,000 in solution¹. However, many biological systems of interest are larger than this and are not amenable to study by conventional NMR techniques because signals within the spectra overlap. Several approaches have been proposed²⁻¹⁰ to simplify such complicated proton NMR spectra by selectively detecting only those protons which are attached to isotopically labelled nuclei. Using these isotope-editing techniques, the proton NMR spectra of large molecular systems can be simplified, and thus made easier to interpret.

One such procedure is a proton spin-echo experiment^{2,3} in which a 180° pulse is applied at the heteronuclear (^{13}C , ^{15}N) frequency on alternate scans concurrent with the proton 180° refocusing pulse. The additional heteronuclear pulse inverts the sign of the proton magnetization of the protons coupled to the labelled nucleus, but has no effect on those protons not coupled to the heteronucleus. Subtracting the spectra that have been acquired with the 180° heteronuclear pulse from those acquired without the pulse reveals only those protons which are coupled to the labelled heteronucleus.

We have recently applied these isotope-filtering techniques to the structure of enzyme/inhibitor complexes¹¹⁻¹³. Figure 1 exemplifies the dramatic spectral simplification that can be achieved using these methods. Compared to the abundance of overlapping resonances observed in the conventional NMR spectrum (Fig. 1c) of a pepsin/inhibitor (Fig. 1a) complex ($M_r \approx 35,000$), only three proton NMR signals (Fig. 1b) of the bound inhibitor — corresponding to those protons attached to the ^{15}N -labels — are detected¹¹. (The large signal corresponds to residual water.)

The spectral simplification that can be achieved using these techniques facilitates the analysis of detailed structural information on large enzyme/inhibitor complexes. For example, the individual NH exchange rates of the bound pepsin inhibitor shown in Fig. 1a were measured¹¹ by tracing the disappearance of the labelled amide proton signals in the simplified spectra (Fig. 1b) as a function of time after the preparation of a D_2O solution of the complex. The NH exchange rates allow the determination of the relative solvent accessibility of each amide proton of the

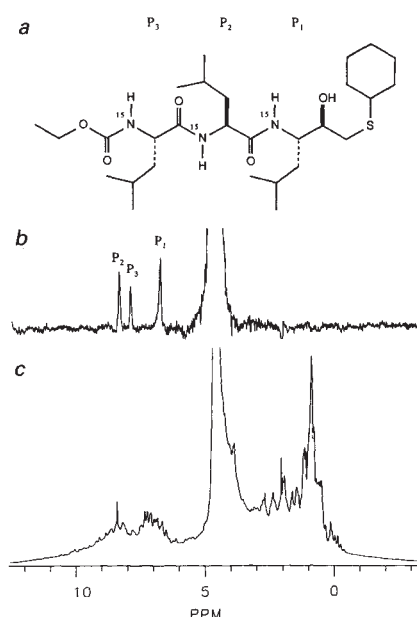


Fig. 1 a, Structure of the labelled pepsin inhibitor. b, Isotope-edited proton NMR spectrum of a 1 mM pepsin/inhibitor (1:1) complex in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1)¹¹. c, Conventional proton NMR spectrum of the complex.

enzyme-bound inhibitor, and may also be used to identify which individual amides of the inhibitor are involved in hydrogen bonding to the enzyme¹¹.

The conformation of enzyme-bound ligands can also be determined using isotope-editing techniques. This has been accomplished¹³ by using nuclear Overhauser effect (NOE) spectra which have been simplified through isotope-editing to identify the protons of the ligand that are in close proximity to one another. Figure 2 depicts part of a contour map from an isotope-filtered two-dimensional NOE spectrum of the enzyme-bound ^{15}N -labelled inhibitor shown in Fig. 1a. The NOE cross-peaks in the spectrum identify the other protons of the ligand that are close to the labelled amide (P_1 – P_3) protons. From an analysis of these NOEs, and those observed in ^{13}C -isotope-edited 2-dimensional NOE spectra of ^{13}C -labelled inhibitors bound to pepsin, the backbone and side-chain conformations (at the P_2 and P_3 sites) of the bound inhibitor were determined¹³.

The structure of the active site can also be elucidated using isotope-edited NOE experiments. NOEs between inhibitor protons attached to isotopically labelled nuclei and enzyme protons identify those protons of the enzyme which are near the

protons of the inhibitor. Although it is difficult unambiguously to assign these inhibitor/enzyme NOEs to one another without performing additional experiments using isotopically labelled enzyme, unassigned NOEs can be of value to test whether other inhibitors bind to the same site on the enzyme. When the NOE data can be combined with structural information from other sources (such as X-ray crystal structures of the enzyme alone or of similar enzyme/inhibitor complexes, or molecular models) the structure of the active site may be characterized in detail, as recently demonstrated for a pepsin/inhibitor complex¹³.

The principal advantages of this approach to studying enzyme/inhibitor complexes are the ease of sample preparation, the ability to use relatively impure enzyme (because only the signals of the labelled inhibitor are observed), and the speed with which the NMR data can be analysed. However, the method does have some limitations. The technique requires isotopically labelled inhibitors and relatively large amounts of protein

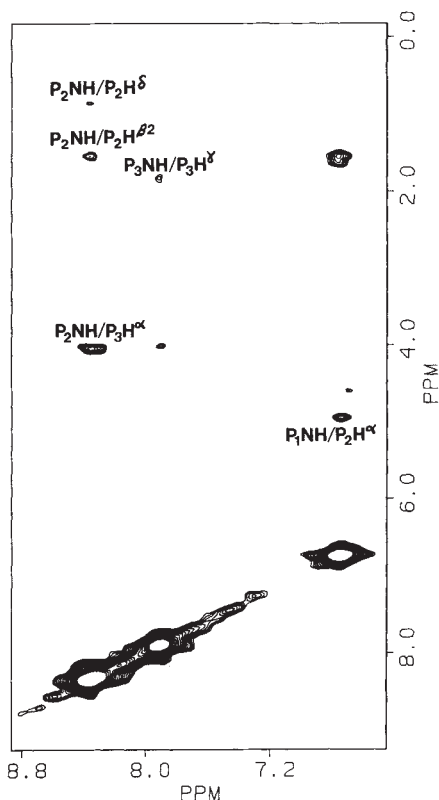


Fig. 2 Portion of a contour plot of an isotope-edited 2-dimensional NOE spectrum of a 1 mM pepsin/inhibitor (1:1) complex¹².

(16 mg in 0.5 ml of solution for pepsin). In addition, even though the spectra are markedly simplified by this technique, spectral overlap may still be a problem if multiple labels are employed. In these cases, however, it may be possible to resolve the overlapping peaks by editing 2-dimensional NMR spectra in a third dimension using the heteronuclear frequencies in a three-dimensional NMR experiment¹⁴.

The potential applications of these techniques are vast. In one application, the structural information that can be obtained from enzyme/inhibitor complexes through isotope-editing may aid in the design of nonpeptide enzyme inhibitors, eliminating the poor absorption and proteolytic instability inherent in peptide inhibitors. The ability to measure NH exchange rates will help to improve the inhibitor by identifying the amides of the inhibitor that are not involved in binding to the enzyme, and that can be removed or altered. Information on the conformation of enzyme-bound inhibitors can be used to suggest new analogues which contain metabolically stable structural frameworks designed to orient spatially the functional groups of the inhibitor for maximum interaction with the enzyme. Finally, knowledge of the structure of the active site will suggest ways to modify inhibitors to fit better into the active site, or ways to change undesirable physical properties of the inhibitor (such as where to attach hydrophilic groups to improve water solubility) without altering the binding affinity of the inhibitor. Indeed, the structural information that can be obtained on the interactions of labelled inhibitors with enzymes or soluble receptors from isotope-editing techniques should be very useful in the design of therapeutic useful pharmaceutical agents.

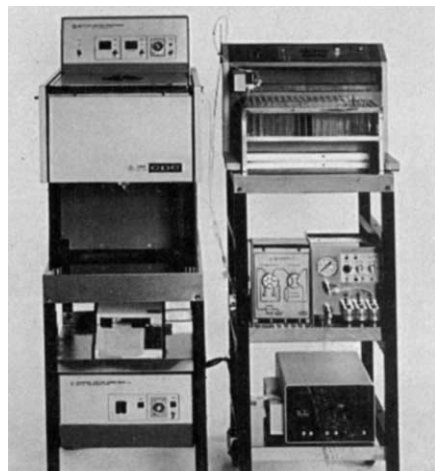
Stephen W. Fesik is at the Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, Illinois 60064, USA. For more information, fill in reader service number 100.

1. Wuthrich, K. *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986).
2. Freeman, R., Mareci, T.H. & Morris, G.A. *J. magn. Reson.* **42**, 341-345 (1981).
3. Bendall, M.R., Pegg, D.T., Doddrell, D.M. & Field, J.J. *Am. chem. Soc.* **103**, 934-936 (1981).
4. Griffey, R.H. & Redfield, A.G. *J. magn. Reson.* **65**, 344-347 (1985).
5. Griffey, R.H., Jarema, M.A., Kunz, S., Rosevear, P.R. & Redfield, A.G. *J. Am. chem. Soc.* **107**, 711-712 (1985).
6. Wilde, J.A., Bolton, P.H., Stolorow, N.J. & Gerlt, J.A. *J. magn. Reson.* **68**, 168-171 (1986).
7. Otting, G., Senn, H., Wagner, G. & Wuthrich, K. *J. magn. Reson.* **70**, 500-505 (1986).
8. Rance, M., Wright, P.E., Messerle, B.A. & Field, L.D. *J. Am. chem. Soc.* **109**, 1591-1593 (1987).
9. Bax, A. & Weiss, M.A. *J. magn. Reson.* **71**, 571-575 (1987).
10. Fesik, S.W., Gampe, R.T., Jr., & Rockway, T.W. *J. magn. Reson.* **74**, 366-371 (1987).
11. Fesik, S.W., Luly, J.R., Stein, H.H., & BaMaung, N. *Biochem. biophys. Res. Commun.* **147**, 892-898 (1987).
12. Fesik, S.W. et al. in *Proceedings of the 10th American Peptide Symposium* (ed. Marshall, G.R.) 57-59 (ESCOM, Leiden, 1988).
13. Fesik, S.W., Luly, J.R., Erickson, J.W. & Abad-Zapatero, C. *Science*, submitted.
14. Fesik, S.W. & Zuiderweg, E.R.P. *J. magn. Reson.*, (in the press).

FASEB in Las Vegas

Next week, over 16,000 scientists will travel to Las Vegas, Nevada for the annual meetings of the Federation of American Societies for Experimental Biology and the American Society for Biological Chemistry and Molecular Biology. Selections from the more than 450 companies with exhibit booths are described below.

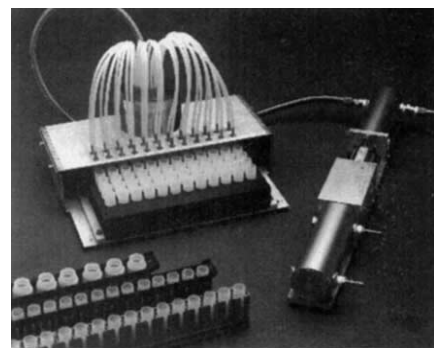
In booths 411 and 413, Sanki Laboratories will be introducing its preparative/analytic **centrifugal partition chromatography** system, the model LLN-5 (*Reader Service No. 101*). Centrifugal partition chromatography (CPC) is a liquid-liquid chromatographic technique which utilizes liquid-liquid partition and counter-current distribution to fractionate complex mixtures of chemical substances. Because no solid support matrix is used in CPC, irreversible retention of highly retentive



Sanki's centrifugal partition chromatograph.

sample components is eliminated. The stationary phase is retained in the column by centrifugal force, so high mobile phase flow rates may be used without appreciable loss of resolution. Sanki says the \$25,000-\$55,000 (US) model LLN-5 CPC system can be used to separate and purify a broad range of synthetic and naturally occurring chemical species, and offers particular advantages in the isolation of polar substances.

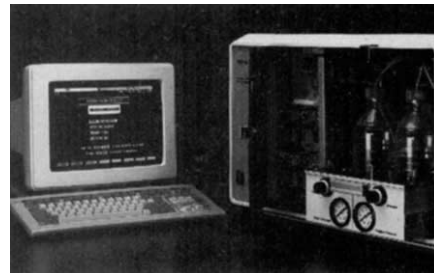
Cambridge Technology, Inc. will be showing off its new model 2600 series **scintillation cocktail distribution pumps** in booth 1435 (*Reader Service No. 102*). The pumps permit up to 24 vials to be filled with 6 ml of any kind of liquid scintillation counter cocktail in approximately 2 seconds, says the company. The semi-automatic system can distribute cocktail from any size or shape of container. The units tie into a central vacuum system or small laboratory vacuum pump, and are operated using a foot pedal. Distribution heads are available for most major models of liquid scintillation counters and



A pump for filling scintillation cocktail vials.

Cambridge Technology's cell harvester vial trays. The \$1,850 (US) price of the pumps includes a choice of distribution heads and foot pedals.

All available synthesis chemistries can be used with the Microsyn 510 **DNA synthesizer** from Perkin-Elmer Cetus, on exhibit in booths 738-841 (*Reader Service No. 103*). Using the beta-cyanoethyl phosphoramidite chemistry, the company says the fully automatic Microsyn 510 produces oligonucleotides at coupling yields of 98.5 per cent per step or better, with minimal depurination. The synthesizer has a patented syringe delivery system which ensures the production of reproducible DNA sequences, and which provides proportional delivery of phosphoramidite bases for the preparation of mixed probes. The \$23,500 (US) Microsyn 510 also has a built-in safety feature to prevent exposure of the user to pressur-



A DNA synthesizer that uses all chemistries.

ized reagent bottles, and a self-monitoring system which automatically detects and signals system failures, low gas levels or empty reagent bottles.

For detecting viral infections in laboratory mice and rats, Organon Teknika offers a range of **animal diagnostic test kits**

(Reader Service No. 104). The tests are based on the principles of solid phase ELISA and require no special reading equipment. Viral antigen, attached to ferrous metal beads, captures specific antibody if present in the test serum. The beads are then incubated with peroxidase conjugated anti-mouse antibodies, and a final incubation with substrate yields a green colour in positive specimens. Among the range of viruses that may be detected are Sendai virus, hepatitis (MHV), pneumonia (PVM), reovirus type 3 and rat coronavirus (RCV/SDA). Each £415 (UK) kit provides 94 determinations; combined test kits which detect four different viruses are also available. Organon Teknika will be exhibiting in booth 261.

Dagan Corporation, in booth 1454, has a new **integrating patch clamp** in which the headstage resistor has been replaced with

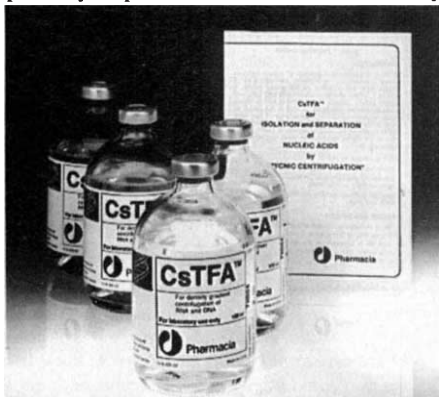


Integrating patch clamp from Dagan.

a capacitor (Reader Service No. 105). Dagan says this design provides 1,000 times better frequency response and substantial noise reduction, and that additional benefits include the absorption of very large current spikes and the simplification of series resistance and capacity compensation controls. The model 3900 patch clamp can be interfaced with any computer because all modes, modifiers, gain, and auto search can be controlled remotely. An optional module specifically for planar lipid bilayers expands the capacity compensation range, delivers a triangle wave and has a fast charge system to charge the bilayer. Dagan's integrating patch clamp costs \$4,900 (US).

Sampling and analysis

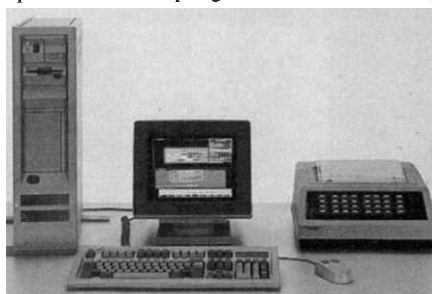
The use of intact undegraded mRNA is of primary importance to the success of any



Pharmacia's solution for isolating RNA.

cDNA library construction. For the preparation of high yields of intact polyadenylated mRNA, Pharmacia LKB, exhibiting in booths from 1116 to 1421, offers **cesium trifluoroacetate solution** (CsTFA) for use in the centrifugation step in the guanidinium thiocyanate/CsTFA procedure of Okayama-Berg (Reader Service No. 106). Pharmacia LKB says DNA fragments, which could interfere with cDNA cloning, are removed completely. CsTFA solution solubilizes and dissociates proteins from nucleic acids without the need for detergents, and is crucial to applications where reduction of unpleasant phenol extraction or lengthy proteinase digestions is the objective. It is also useful for the isolation of both single and double-stranded RNA and DNA from cells, viruses and organelles.

ChromStation/2 is the newest **chromatography workstation** from Spectra-Physics (Reader Service No. 107). The \$6,900 (US) ChromStation/2 couples a multichannel data system with a LC system controller. Designed for the IBM PS/2 series of computers, the workstation performs tasks from collecting and storing data to reintegrating and writing up the results in a spreadsheet. With ChromStation/2, the chromatographer is able to operate several programs simultaneously



Chromatography with the IBM PS/2 from Spectra-Physics.

using a multitasking operating environment; switching between programs is accomplished with a click of the mouse. Equipped with a Spectra-Physics Labnet communications interface, the ChromStation/2 can collect data automatically from up to eight channels, store it on disk, and transfer it to other chromatography software programs. Spectra-Physics will be showing its full line of chromatography systems, including the ChromStation/2, in booths 111 and 113.

The Ultrafree-MC filter unit and **personal centrifuge** from Millipore are intended for sample preparation of micro-litre volumes of solution prior to HPLC and ion chromatography analysis (Reader Service No. 108). The Ultra-free-MC filter unit is designed for the removal of cells and particulates, and for the purification and recovery of proteins, enzymes and virus. It is available with both ultrafiltration and low-binding microporous membranes,

and the unit's standard 1.5 ml conical tube also fits conventional centrifuges. The \$350 (US) Millipore personal centrifuge is smaller than a standard stir plate, and can be operated in a laminar flow hood, a refrigerator, or other crowded areas. There are no timers to set and no switches to operate. Millipore is exhibiting in booths 186-287.

The gel lane

Molecular Dynamics will be unveiling a new development in **densitometry** in booth 275 (Reader Service No. 109). The company's model 300A Computing Densitometer 'reads' a gel or an autoradiograph in the same way that an optical scanner reads a printed page. The densitometer uses a line scanner and an integrating cylinder to analyse the entire gel area at once, not just individual lanes. The sample moves in one direction, while the light beam moves in a perpendicular direction, so the densitometer scans every data point. Molecular Dynamics says the model 300A can analyse a 36 cm x 43 cm gel in just three minutes, and that the light integrating cylinder collects virtually all of

ADVERTISEMENTS

Economical Modular Tissue Culture Incubator

- Safe for AIDS research • Reliable, for In-Vitro fertilization
- Versatile enough for thousands of lab uses

Easy to use - Just flush with gas mixture, seal and place at approp. temp. Perform multiple experiments simultaneously w/out cross contamination. Thousands in use worldwide.

For information contact: **Flow Laboratories, Ltd.** or **Billups-Rothenberg, Inc.** • P.O. Box 977
Del Mar, CA 92014-0977 USA • (619) 755-3309

Reader Service No.25

SINGER
INSTRUMENTS

MICROINJECTION?

- SINGER MKI
- SINGER MKIII
- SINGER MICROFORGE

SINGER INSTRUMENT CO LTD
TREBOROUGH LODGE
ROADWATER, WATCHET
SOMERSET TA23 0QL
Tel: 0984-40226
Telex: 337492 COMCABG

Reader Service No.50



A new slant on densitometry.

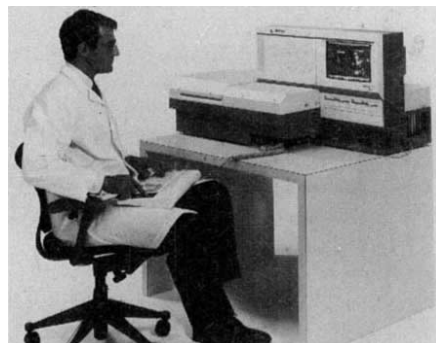
the transmitted light so accurate measurements can be made even in the 3 to 4 optical density range. The system's computer uses Windows-based software, allowing the results from up to four gels to be viewed simultaneously for comparison. Molecular Dynamics sells the model 300A Computing Densitometer for \$29,500 (US).

Bio-Rad will have selections from its product range on display in booths 1043–1050. New from Bio-Rad are **sequencing wedge spacers** for its 40- and 50-cm long Sequi-Gen DNA sequencing cells (*Reader Service No. 110*). Wedge spacers produce more closely spaced bands at the bottom of the gel, increasing the number of readable bases per lane. The 40-cm long spacers form gels 0.25 to 0.53 mm thick, and the 50-cm long spacers form gels 0.25 to 0.75 mm thick. Wedge spacers eliminate the need for buffer gradient gels, allowing the use of standard polyacrylamide solutions and buffers for sequencing.

Bethesda Research Laboratories, exhibiting in booths from 1656 to 1757, has recently announced the launch of its Gel-Mix 6 **pre-mixed gel-casting solution** for the preparation of sequencing gels (*Reader Service No. 111*). The six per cent polyacrylamide solution includes TBE

buffer, urea and TEMED, and BRL says it remains stable for six months. Preparation of gels using the solution is easy: researchers just add ammonium persulfate to the bottle, mix it for 30 seconds, and pour the solution into the gel mould. The Gel-Mix 6 comes in packages of six 75-ml bottles; each bottle makes a standard sized 33 cm × 40 cm × 0.04 cm gel.

The Kodak company named Bio Image will be displaying its range of machine vision **image analysis instruments for gel electrophoresis** in booths 1244 and 1343. Bio Image offers a benchtop analyser the



Benchtop gel analysis from Bio Image.

size of a densitometer, the Visage BT (*Reader Service No. 112*). The Visage BT acquires images by transmittance or reflectance using a 512 × 1 solid state photodiode array. The system offers selectable 50 μ per pixel or 100 μ per pixel resolution. The CPU of the system is an Intel 80286 16-bit microprocessor which supports MS-DOS, and images are displayed on a 12-inch high resolution monitor. The analytical capabilities of the Visage BT include 1-dimensional electrophoresis quantification and 2-dimensional spot quantification.

Reagents and kits

Two new products for the use and handling of **chromogens for peroxidase and pseudo-peroxidase reactions** are available from ICN Biomedicals, Inc., exhibiting in booths 1124–1132 (*Reader Service No. 113*). ICN Biomedicals offers both a soluble chromogen solution, TMB, for ELISA and an insoluble chromogen solution, 4-chloro-1-naphthol, for immunohistology procedures. The two substrates are available in ready-to-use solution form, eliminating the need to make up new chromogens for each enzyme reaction. The chromogens are combined with peroxide and stabilizers so that they can be used directly as the final step in developing the colour indicator in the enzyme reaction. ICN Biomedicals sells the chromogen/substrate solutions for \$110 (US) for 50 ml.

Molecular Biology Resources, in booth 1666, will be introducing its immuno-affinity purified **human DNA polymerase α-primease** (*Reader Service No. 114*). DNA polymerase-α is a key component in the chromosomal replicating apparatus of higher eukaryotes, and MBR's DNA polymerase-α should be useful in studies requiring reconstitution of model eukaryotic DNA replicating systems. The polymerase is analysed for contaminating exo and endonucleases, and is functionally tested in a DNA polymerase α-primease-dependent M13 replication system. MBR sells the DNA polymerase α-primease in 50 and 200 unit packages, for \$84 and \$310 (US), respectively.

Software

Recombinant Toolkit is the name of the newest software package from Biosoft, exhibiting in booth 1579 (*Reader Service No. 115*). Recombinant Toolkit is a **DNA and amino acid sequence analysis package** compatible with IBM PCs equipped with a colour monitor. The \$350 (US) software finds restriction sites for approximately 100 restriction enzymes, performs sequence searches and translates DNA sequences to peptide/protein sequences. Special functions include: codon counts for checking the validity and nature of active sequences, start/stop/renumbering facilities for defining a subsequence of an active sequence or for renumbering sequences, and the ability to modify tables of restriction enzymes or genetic codes. The hydropathy feature illustrates protein sequences of up to 700 amino acids using colour gradients to indicate the moving average or hydrophobic moment. □

These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

ADVERTISEMENT

Introducing:

Hyaluronic Acid Test

Hyaluronic acid (hyaluronan) is present in most tissues but is especially abundant in loose connective tissues. It is released and transported via the blood to the liver where it is catabolized. Increased levels have therefore been de-

tected in patients with e.g. rheumatoid arthritis and liver cirrhosis.

Pharmacia offers you a sensitive, specific and reproducible radioassay for measurement of hyaluronic acid.

For research use only. Not for use in diagnostic procedures.

Pharmacia Diagnostics
Division of Electro-Nucleonics, Inc.
Attn: Gary Britton
368 Passaic Avenue
FAIRFIELD, NJ 07006, USA
Phone 1-800-526-3625

Pharmacia Diagnostics AB
Attn: Ulf Almqvist
S-751 82 Uppsala
Sweden
Phone 46-18-16 39 00